ESTIMATION OF COTININE LEVEL IN THE SERUM AND SALIVA IN ACTIVE SMOKERS AND NON-SMOKERS

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CERTIFICATE

This is to certify that this dissertation titled "**ESTIMATION OF COTININE LEVEL IN THE SERUM AND SALIVA IN ACTIVE SMOKERS AND NON-SMOKERS**" is a bonafide record of work done by **Dr. S. Parthiban** under my guidance during his postgraduate study period **2010-2013.**

This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY,** in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY, BRANCH IX – Oral Medicine & Radiology.**

It has not been submitted (partial or full) for the award of any other degree or diploma.

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LIST OF ABBREVIATIONS

S.NO	ABBREVIATION	EXPANSION
1.	AIDS	Acquired Immuno Deficiency Syndrome
2.	CM	Carbon Monoxide
3.	COHb	Carboxy Hemoglobin
4.	ETS	Environmental Tobacco Smoke
5.	GC	Gas Chromtography
6.	GCF	Gingival Crevicular Fluid
7.	GS-MS	Gas Spectrometry Mass Spectrometry
8.	GLPC	Gas liquid Partition Chromatography
9.	GYTS	Global Youth Tobacco Survey
10.	HCN	Hydrogen Cyanide
11.	IARC	International Agency for Research on Cancer
12.	LC-AP1-MS-MS	Sensitive Atmospheric Pressure Ionization Tandem Mass Spectrometric
13.	LSD	Lysergic Acid Diethylamide
14.	OR	Odds Ratio
15.	PAH	Poly Aromatic Hydrcarbon
16.	PCP	Phencyclidine
17.	PDA	Photo Diode Array

18.	RAST	Radio Allergo Sorbent Test
19.	RIA	Radioimmunoassay
20.	RSD	Relative Standard Deviation
21.	SHS	Second Hand Smoking
22.	TSNA	Tobacco Specific Nitroso Amine
23.	USA	United States of America
24.	VPC	Vapour Phase Chromatography
25.	WHO	World Health Organization

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ABSTRACT

STUDY TITLE: ESTIMATION OF SERUM AND SALIVA COTININE LEVELS IN ACTIVE SMOKERS AND NON SMOKERS

Background and Objectives: Smoking is the single most cause of disability, and death affecting the World's population today. Cotinine the major metabolite of nicotine is generally regarded as the best biomarker for monitoring tobacco exposure in both actively and passively exposed individuals. The aim of the study was to estimate and compare cotinine level in smokers and Non- smokers in saliva and serum by High profile liquid chromatography.

Materials and methods: Serum and Saliva samples were collected. Chromatography was performed using an L-7100 pump, an L-7400 UV detector, an L-7200 auto sampler, an L-7500 integrator and an 865-CO column oven. Cotinine was quantified by comparing the HPLC peak heights to those of authentic standard.

Results: Study group showed higher serum and saliva cotinine levels than control group. Serum cotinine levels were significantly higher than saliva cotinine levels in study group. Serum and saliva cotinine levels in control group was not significant.

Conclusion: Our study shows the importance of estimating cotinine levels for distinguishing tobacco users from non-users, for estimating the nicotine intake of tobacco users and for specifying the exposure of nonsmokers to second hand smoke. The use of High profile liquid chromatography with its superior resolving power is also recommended. However more studies are needed using High profile liquid chromatography to establish standardized values.

Keywords: Cigarette smoke, Cotinine, High profile liquid chromatography

Smoking is the single most cause of disability, and death affecting the World's population today. The World Health Organization (WHO) reports that 15 billion cigarettes are sold daily and that approximately a third of the global male adult population smokes as quoted by (WHO 2002). Globally, one in ten adults die from smoking and related diseases every day and the WHO states that if this current trend continues, by the year 2030, smoking will kill approximately one in six people as per (WHO 2020). Smoking is a global issue and despite progress in reducing smoking prevalence, it is still a huge problem affecting many countries.

The Global Adult Tobacco Survey (GATS) is a nationally representative household survey that was launched in February 2007 as a new component of the ongoing Global Tobacco Surveillance System (GTSS). According to that survey, current tobacco smokers in India are 14.9% in which cigarette smokers are 5.7%. The overall male prevalence is 24.3% out of which current cigarette smokers are 10.3%.

Adults who are exposed to second hand smoke at home are 52.3% and adults exposed to second hand smoke at work place are 29.9% and adults exposed to second hand smoke at public place are 29.0%.[1]

In India, tobacco consumption is responsible for half the number of all the cancers in men and a quarter of all cancers in women, in addition to being a risk factor for cardiovascular diseases and chronic obstructive pulmonary diseases.[2]

The World Health Organization predicts that tobacco deaths in India may exceed 1.5 million annually by 2020.[3] There are at least 55 carcinogens in cigarette smoke, and presently available data focus on 20 substances that are probably involved in lung cancer induction.[4]

Nicotine is named after the tobacco plant Nicotian Tabacum, which in turn is named after Jean Nicot de Villemain, French Ambassador in Portugal, who sent tobacco and seeds from Brazil to Paris in 1560 and promoted their medicinal use. Nicotine was first isolated from the tobacco plant in 1828 by physician Wilhelm Heinrich Posselt and chemist Karl Ludwig Reimann of Germany, who considered it a poison.[5]

Cotinine is a useful and popular biomarker of tobacco use. Most nicotine entering the body (70%–80%) is metabolized into cotinine. Cotinine is present in the blood serum, saliva, urine, amniotic fluid, cervical mucus and hair of both smokers and non-smokers exposed to tobacco smoke. It has been cited as the most useful marker for distinguishing tobacco users from non-users, for estimating the nicotine intake of tobacco users and for specifying the exposure of nonsmokers to second hand smoke.[6]

Cotinine has an extended biological half-life of 15 to 40 hours. Its level in the body is directly related to the quantity of nicotine absorbed during the last few days.[7] The presence of cotinine indicates exposure to nicotine, either from environmental exposure or direct consumption.

High-performance liquid chromatography (sometimes referred to as high-pressure liquid chromatography), HPLC, is a chromatographic technique used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying and purifying the individual components of the mixture. HPLC is also considered an instrumentation technique of analytical chemistry, instead of a gravimetric technique. HPLC has many uses including medical (e.g. detecting vitamin D concentrations in blood serum), legal (e.g. detecting performance enhancement drugs in urine), research (e.g. purifying substances from a complex biological sample, or separating similar synthetic chemicals from each other) and manufacturing (e.g. pharmaceutical quality assurance).[8]

AIM OF THE STUDY:

To estimate and compare cotinine level in smokers and non-smokers in serum and saliva.

OBJECTIVE OF THE STUDY:

- To estimate serum cotinine level in non-smokers.
- To estimate saliva cotinine level in non-smokers.
- To estimate serum cotinine level in smokers.
- To estimate saliva cotinine level in smokers.
- To compare serum and saliva cotinine level in non-smokers and smokers.

SMOKING

HISTORY

The history of smoking starts among the Native Americans who used it for ceremonial purposes 5000 years BC. Christopher Columbus first brought tobacco to Europe from the West Indies in 1492. From the beginning it was used for medical purposes and in history it is mentioned when the Queen of France, Catherine of Medici, was cured from stomach pains by tobacco. She got the tobacco from Jean Nicot and named it "Nicotiana". Soldiers during the great European wars spread the use of tobacco, mostly used as snuff or smoked in pipes. It was not until the Crimean War, in the middle of 19th century, that cigarettes became more common. When the first cigarette machine was constructed in 1870, cigarette smoking flourished. This was also the start for the big tobacco company.[9]

Cigarette smoke is a complex mixture of chemicals. Some smoke components, such as carbon monoxide (CO), hydrogen cyanide (HCN) and nitrogen oxides, are gases. Others, such as formaldehyde, acrolein, benzene, and certain N-nitrosamines, are volatile chemicals contained in the liquid-vapor portion of the smoke aerosol. Still others, such as nicotine, phenol, polyaromatic hydrocarbons (PAHs) and certain tobacco-specific nitrosamines (TSNAs), are contained in the submicron sized solid particles that are suspended in cigarette smoke.

In view of this chemical complexity, cigarette smoke has multiple, highly diverse effects on human health. It is not unexpected that multiple chemicals in cigarette smoke can contribute to any single adverse health effect.[10]

EPIDEMIOLOGY[11]

Though smoking prevalence in the western world is decreasing, smoking has kept an aura of tough and smart glamour, and around 10,000 new young smokers are recruited daily. In total, about 1/3 of the adult population smokers and WHO has calculated that 1000 cigarettes are manufactured per year per person, including women and children. An early two-fold difference in smoking rates is seen in men across different WHO regions, with the lowest level in the Eastern Mediterranean Region (34.2%) and the highest in the Western Pacific Region (62.3%). Based on these weighted prevalence estimates, there are over 1.2 billion smokers across the six WHO regions, women being in the minority in the developing countries.

Tobacco use prevalence can be decreased by a variety of tobacco prevention and control efforts. Reporting on the adverse health effects from smoking the anti-smoking debate was accelerated in the 1980's when it was shown that passive smoking was also a health hazard. During the 1990's numerous conventions, national as well as international, addressed the smoking issue. Educational, clinical, regulatory, economic and comprehensive approaches are widely used and studied. WHO and European Union, have

made up rules and recommendations for how the "pandemic of smoking" can be defeated. Tobacco control is highly cost effective. Many countries have passed laws on smoke free areas, rules for cigarette commerce and public health interventions to control tobacco use.[12]

As an example in Finland, the Tobacco Control Act was passed as early as in 1976. It prohibited smoking in most public places, restricted tobacco advertising, and set a 16-year age limit for tobacco purchases. Further amendments to the Act were made in 1995, when, for example, the age limit for tobacco purchases was raised to 18 years, and in 2000, when ETS was included in the national list of carcinogenic substances. Among Finnish adult males, smoking prevalence is nowadays one of the lowest in Europe. In general, the smoking trends suggest that the impact of tobacco policy is decreasing smoking initiation in youth, for example the legislation appears to have decreased purchases from commercial sources to minors.[13]

FORMS OF TOBACCO

There is a variety of smoking tobacco products on the world market.

1. **Cigarette** is any roll of tobacco wrapped in paper or other non-tobacco material wrapped in paper with filter-tipped or untipped approximately 8 mm in diameter, 70–120 mm in length.

2. **Cigar** is any roll of tobacco wrapped in leaf tobacco or in any other substance containing tobacco. There are four main types of cigars.

a. **Little cigars** contain air-cured and fermented tobacco and are wrapped either in reconstituted tobacco or in a cigarette paper that contains tobacco and/or tobacco extract.

b. **Small cigars** or Cigarillos are small, narrow cigars with no cigarette paper or acetate filter.

c. **Regular cigars** are up to 17 mm in diameter, 110-150 mm in length.

d. **Premium cigars** (hand-made from natural, long filter tobacco) vary in size, ranging from 12 to 23 mm in diameter and 127 to 214 mm in length.[14]

Cigarettes and cigars use blended tobaccos and the type of tobacco used in these products has a decisive influence on the physicochemical nature of the smoke they produce.

3. **Bidis** are the most popular form of smoking of tobacco in India. They are also becoming increasingly popular among teenagers in the USA. A bidi is made by rolling a rectangular piece of a dried temburni leaf around approximately 0.2–0.3g of sun-dried, oriental tobacco and securing the roll with a thread. This type of smoking is perceived by some as better tasting, cheaper, safer or more natural alternative to conventional cigarettes.[15]

4. **Chuttas** are coarsely prepared cheroots with 2–9 cm long, prepared by rolling local tobacco inside a sun-dried tobacco leaf. They are usually the products of cottage or small-scale industries. Nearly 9% of the tobacco produced in India is used for making chuttas. It is estimated that about 3000 million chuttas are made annually in India. The term "reverse smoking" is used to describe smoking while keeping the glowing end of tobacco product inside the mouth. Reverse chutta smoking is practised extensively by women in the rural areas of Visakhapatnam and the Srikakulam district of Andhra Pradesh.[16]

5. **Cheroot** is a roll made from tobacco leaves. Cheroots were commonly smoked by both Indian men and women in South India.

6. **Dhumti** is a kind of conical cigar made by rolling tobacco leaf in the leaf of another plant. Unlike bidis and chuttas, dhumtis are not available from vendors but are prepared by the smokers themselves.[17]

7. **Kreteks** are types of small cigarettes that contain tobacco (approximately 60%), ground clove buds (40%) and cocoa, which gives a characteristic flavour and "honey" taste to the smoke. Kreteks are indigenous to Indonesia, but are also available in the USA.[14]

8. **Pipe** smoking is one of the oldest form of tobacco use. The different kinds of pipes used for smoking range from the small – stemmed

European types made of wood to long-stemmed pipes made from metal or other material.

9. **Hookah** is an Indian white pipe in which the tobacco smoke passes through water before inhalation. It used to be more common among women, the reason being that it was inconvenient for men to carry a hookah, whereas women remain at home for most of the time.

10. **Hooklis** are clay pipes commonly used in Western India. Once the pipe is lit, it is smoked intermittently. On average, 15 g of tobacco is smoked daily. Hookli smoking was common among men in the Bhavnagar district of Gujarat.[18]

11. **Chillum** is a straight conical pipe made of clay, 10–14 centimeters long, held vertically. It is exclusive and common among men and is confined to the northern states of India, predominantly rural areas.[19]

BIOCHEMICAL METHODS

The term biomarker means a measurement that reflects an interaction between a biological system and a chemical, physical, or biological environmental agent. Biological quantification of tobacco use is based on some aspect of the composition of inhaled tobacco smoke. Tobacco smoke is composed of gaseous and particle components. The gaseous component is made up of room air, carbon monoxide, nicotine and volatilized hydrocarbons such as hydrogen cyanide. The primary particle component of tobacco smoke is tar, which carries nicotine. Substances such as nicotine, cotinine, thiocyanate, carbon monoxide and some minor alkaloids of nicotine have been

identified and tested as biomarkers of both active cigarette smoking and second hand smoke exposure (SHS).[20]

NICOTINE

The major and most pharmacologically active alkaloid of tobacco is nicotine. The amount of nicotine uptake is dependent on a smoker's inhalation behaviours (e.g deep or long inhalation of smoke) and metabolism of nicotine. Most nicotine is metabolized into cotinine and eventually excreted (see cotinine below). Nicotine may be extracted and measured from blood, saliva, and urine.[21]

More recently. It has been measured from samples of hair and toenails. Nicotine as a biomarker agent, however, is of limited use. Any assay using nicotine must be very sensitive because of the small amount of nicotine present in body fluids. Furthermore, because of its short half-life (2 hrs) and individual variation in its rate of metabolism, nicotine levels can be only approximated, and may give a biased estimate of tobacco use/exposure.

THIOCYANATE

Tobacco smoke contains high concentrations of hydrogen cyanide gas, which is primarily metabolized into thiocyanate (SCN). Like cotinine, SCN can be measured in blood, urine and saliva. The following issues affect the usefulness of SCN as a biomarker. Though SCN has longer half-life (10-14 days), the sensitivity and specificity of the assay method are low. SCN levels are influenced by industrial exposure and dietary intake of substances like almonds, bamboo shoots, sugar cane, cauliflower, broccoli and beer.

Because of these limitations, determination of SCN has not gained wider use.[22]

CARBON MONOXIDE

Cigarette smoke contains a high concentration of CO in gaseous form. Regular cigarette smoking may produce carboxyhemoglobin (COHb) levels ranging from 5% (1 pack per day) to 9% (2-3 packs per day), whereas heavy cigar smoking can produce COHb levels up to 20%. CO has a half-life of 4-5 hrs in adults and can be measured in both exhaled alveolar air and blood as stated by Stewart 1975. Although CO can be measured by analysis of hemoglobin for COHb using a carbon monoxideoximeter instrument, this approach is not favoured because the procedure to collect the specimen (blood) is invasive. Instead, a much simpler and direct measurement of CO can be accomplished using exhaled air and a simple handheld breath analyzer. This method does not require the samples, such as those of blood, saliva, or urine, to be collected and stored, and only minimal training is needed in using the device. The immediately available measurement of CO level, which is shared with the smoker, can depict the detrimental effects of smoking. This may affect the smoker's subsequent smoking behavior.[23]

Thus, CO measurement has been used as part of anti-smoking campaigns. Researchers have demonstrated high correlations among CO, self-reported smoking and urinary cotinine as said by Secker-Walker et al. in 1997. Exhaled CO has been successfully used to corroborate self-report data, with concordance approaching 100%.

Environmental sources of CO can result in CO levels indistinguishable from those produced by direct cigarette use, thereby confounding the measurement another disadvantage of CO measurement is the relatively short half-life of CO (4-5 hrs). In general population, false-negative rates of CO measurements have been found to range from 2% to 16%. In addition, the sensitivity decreases with infrequent and irregular smoking patterns, causing those who are light or atypical smokers to appear indistinguishable from non-smokers.[24]

A REVIEW OF SALIVA AS DIAGNOSTIC FLUID[25]

Saliva, the most available and non-invasive bio-fluid of the human body, permanently "bathes" the oral cavity and is trying to cope with an ever-changing milieu. The oral cavity, a very complex and unique milieu due to its dual function, is the only place in the body where the mineralized tissue is exposed to the external environment in which there are complex interactions between various surfaces such as host soft and hard tissues, food, air and microorganisms. Saliva includes a large number of inorganic and organic compounds, which act as a "mirror of the body's health". In addition to its other functions, saliva could constitute the first line of defense against oxidative stress. Due to its composition and functions, saliva could have a significant role in controlling and/or modulating oxidative damages in the oral cavity. As a diagnostic fluid, saliva offers distinctive advantages over serum. Furthermore, saliva may provide a cost-effective approach for the screening of

large populations. Gland-specific saliva can be used for diagnosis of pathology specific to one of the major salivary glands. Whole saliva, however, is most frequently used for diagnosis of systemic diseases.

As we enter the era of genomic medicine, sialochemistry will play an increasingly important role in the early detection, the monitoring and progression of the systemic and oral diseases. We reviewed the current data within literature and of our research concerning clinical potential of the saliva.[25]

Saliva is derived from several types of salivary glands. Each type of salivary gland secretes saliva with characteristic composition and properties. The secretions from these different glands have been shown to differ considerably, to be complex in composition and to be affected by different forms of stimulation, time of day, diet, age, gender, a variety of disease states, and several pharmacological agents. Whole saliva is a mixed fluid that is derived predominantly from 3 pairs of major salivary glands: the parotid, the submandibular and the sublingual glands. Approximately 90% of total salivary volume results from the activity of these 3 pairs of glands, with the bulk of the remainder from minor salivary glands located at various oral mucosal sites. The whole saliva also contains gingival crevicular fluid (GCF), mucosal transudations, expectorated bronchial and nasal secretions, serum and blood derivatives from oral wounds, bacteria, and bacterial products, viruses and fungi, desquamated epithelial cells, other cellular components and food debris.

Serum constituents that are not part of the normal salivary constituents (i.e., drugs and hormones) can reach saliva by several ways: intracellular (through passive transfer, by diffusion) and extracellular (ultrafiltration).

Serum constituents are also found in whole saliva as a result of GCF outflow. Depending on the degree of the inflammation in the gingiva, GCF is either a serum transudation or more commonly, an inflammatory exudation that contains serum constituents. Saliva can be collected with or without stimulation. The best two ways to collect whole saliva are the draining method, in which saliva is allowed to drip off the lower lip, and the spitting method, in which the subject expectorates saliva into a test tube.

Tobacco usage or exposure (via “passive” or “second-hand” smoke) is now routinely measured by quantization of levels of salivary nicotine that are similar clearance and half-life values as plasma. Monitoring levels of salivary nicotine has proven useful in monitoring self-reported compliance with smoking cessation programs. Salivary nicotine levels were found to be indicative of active and passive smoking. Salivary thiocyanate was also found to be an indicator of cigarette smoking; however, nicotine levels are considered the most reliable marker .An adequate intake may help smokers to avoid cigarette smoke induced oxidative damage and to prevent degenerative disease. The smoking causes the decrease in salivary important antioxidants levels and the loss of activity of salivary enzymes with antioxidant actions can be considered as one of the mechanisms by which the toxic effects of CS

initiate oral inflammatory diseases, promote precancerous transformations and destroy the oral cavity homeostasis.

Evaluation of the quantity of whole saliva is simple and may provide information, which has systemic relevance. Quantitative alterations in saliva may be a result of medications (at least 400 drugs may induce xerostomia). Diuretics, antihypertensives, antipsychotics, antihistamines, antidepressants, anticholinergics, antineoplastics, amphetamines, barbiturates, hallucinogens, cannabis, and alcohol have been associated with a reduction in salivary flow and may lead to oral problems like progressive dental caries, fungal infection, oral pain, and dysphagia. Qualitative changes in salivary composition can also provide diagnostic information concerning oral problems: increased levels of albumin in whole saliva were detected in patients who received chemotherapy as treatment for cancer and subsequently developed stomatitis, reduced salivary EGF levels may be important for the progression of radiation-induced mucositis, higher levels of salivary nitrate and nitrite, and increased activity of nitrate reductase, were found in oral cancer patients compared with healthy individuals, and were associated with an increased odds ratio for the risk of oral cancer.[25]

Detection, measurement, and monitoring of drugs many analyses, including drugs of abuse, can be measured in saliva and oral fluids. Particularly useful where a "yes/no" answer is required, oral fluid based tests find wide usage in detection of recreational drugs, including alcohol,

amphetamines, barbiturates, benzodiazepines, cocaine, a variety of inhalants, lysergic acid diethylamide (LSD), marijuana, opioids, phencyclidine (PCP), and tobacco. The use of saliva for drug monitoring, and the detection of illicit drugs, has grown remarkably. Currently, saliva can be used to detect and/or monitor nicotine, cannabinoids, cocaine, phencyclidine, opioids, barbiturates, diazepams, amphetamines and ethanol, most recently, law enforcement agencies have employed saliva-based tests for roadside evaluation of alcohol levels and in hospital emergency departments as a rapid means of determining whether impaired consciousness is related to alcohol intoxication.[25]

COTININE

Cotinine is the major degradation product of nicotine metabolism and has a serum half-life of about 17 hours compared to two hours for the parent compound. Measurement of cotinine levels can provide a sensitive estimate of tobacco smoke exposure. For the purpose of developing epidemiologic studies, comparative data on the relative sensitivities of cotinine measurements in serum, saliva, and urine are required, but few such data are available. In the present study, we compared cotinine levels in samples of serum, saliva and urine in nonsmokers, passive smokers, and active smokers.[26]

Cotinine is a useful and popular biomarker of tobacco use. Most nicotine entering the body (70%–80%) is metabolized into cotinine. Cotinine is present in the blood serum, saliva, urine, amniotic fluid, cervical mucus and hair of both smokers and non-smokers exposed to tobacco smoke. It has been

cited as the most useful marker for distinguishing tobacco users from non-users, for estimating the nicotine intake of tobacco users and for specifying the exposure of nonsmokers to second hand smoke.[6]

Cotinine has an extended biological half-life (15–40 hrs). Its level in the body is directly related to the quantity of nicotine absorbed during the last few days.[7] The presence of cotinine indicates exposure to nicotine, either from environmental exposure or direct consumption.

An advantage of cotinine as a biomarker is its high sensitivity. It can distinguish very low levels, such as from SHS in non-smokers, from levels associated with cigarette smoking. Small amounts of cotinine in the body can result from ingestion of foods rich in nicotine (such as cauliflower, eggplant, potatoes, tomatoes and black tea), but these levels are considered insignificant.[27] Measurement techniques have been developed. Cotinine can be quantified in blood, serum, saliva and urine. Various techniques are used for quantitative analysis including: (a) radio immunoassay, (b) high-performance liquid chromatography, (c) gas–liquid chromatography and (d) gas chromatography combined with mass spectrometry.[28] Woodward and colleagues (1991) compared cotinine levels with those from exhaled CO, self-reported tobacco exposure and thiocyanate. The results showed a high correlation among all the markers for the smoking group, but a lower correlation among the nonsmokers exposed to second hand smoke. The investigators concluded that cotinine is the most accurate discriminator

between smokers and non-smokers as stated by Woodward et al (1991). In other studies, serum cotinine was demonstrated to be a better measure of cigarette smoking than was questionnaire.[29] Exhaled carbon monoxide and cotinine (detected in blood, urine or saliva) are sufficiently sensitive, specific and feasible for general use, and are therefore frequently used as biomarkers of cigarette smoking.

METHODS TO ESTIMATE COTININE LEVELS

High-performance liquid chromatography sometimes referred to as high-pressure liquid chromatography HPLC, is a chromatographic technique used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying and purifying the individual components of the mixture. HPLC is also considered an instrumentation technique of analytical chemistry, instead of a gravimetric technique. HPLC has many uses including medical (e.g. detecting vitamin D concentrations in blood serum), legal (e.g. detecting performance enhancement drugs in urine), research (e.g. purifying substances from a complex biological sample, or separating similar synthetic chemicals from each other) and manufacturing (e.g. pharmaceutical quality assurance).

HPLC relies on the pressure of mechanical pumps on a liquid solvent to load a sample mixture onto a separation column, in which the separation occurs. A HPLC separation column is filled with solid particles (e.g. silica, polymers, or sorbents) and the sample mixture is separated into compounds as

it interacts with the column particles. HPLC separation is influenced by the liquid solvent's condition (e.g. pressure, temperature), chemical interactions between the sample mixture and the liquid solvent (e.g. hydrophobicity, protonation and chemical interactions between the sample mixture and the solid particles packed inside of the separation column (e.g. Ligand affinity, ion exchange.

HPLC is distinguished from ordinary liquid chromatography because the pressure of HPLC is relatively high, while ordinary liquid chromatography typically relies on the force of gravity to provide pressure. Due to the higher pressure separation conditions of HPLC, HPLC columns have relatively small internal diameter (e.g. 4.6 mm), are short (e.g. 25 mm) and packed more densely with smaller particles, which helps to achieve finer separations of a sample mixture than ordinary liquid chromatography can. This gives HPLC superior resolving power when separating mixtures, and hence it is a popular chromatographic technique.

The schematic of an HPLC instrument typically includes a sampler by which the sample mixture is injected into the HPLC, one or more mechanical pumps for pushing liquid through a tubing system, a separation column, a digital analyte detector (e.g. a UV/Vis, or a photodiode array (PDA) for qualitative or quantitative analysis of the separation, and a digital microprocessor for controlling the HPLC components (and user software). Many different types of columns are available, varying in size and in the type

(i.e. chemistry) of solid packed particle types available. Some models of mechanical pumps in a HPLC instrument can also mix multiple liquids together, and the recipe or gradient of those liquids can modify the chemical interactions that occur in HPLC's column, and thereby modify the chemical separation of the mixture.[8]

Gas chromatography (GC), is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance or separating the different components of a mixture (the relative amounts of such components can also be determined). In some situations, GC may help in identifying a compound. In preparative chromatography, GC can be used to prepare pure compounds from a mixture.

In gas chromatography, the mobile phase (or "moving phase") is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column (an homage to the fractionating column used in distillation). The instrument used to perform gas chromatography is called a gas chromatograph (or "aerograph", "gas separator").

The gaseous compounds being analyzed interact with the walls of the column, which is coated with different stationary phases. This causes each compound to elute at a different time, known as the retention time of the

compound. The comparison of retention times is what gives GC its analytical usefulness.

Gas chromatography is in principle similar to column chromatography (as well as other forms of chromatography, such as HPLC, TLC), but has several notable differences. Firstly, the process of separating the compounds in a mixture is carried out between a liquid stationary phase and a gas mobile phase, whereas in column chromatography the stationary phase is a solid and the mobile phase is a liquid. (Hence the full name of the procedure is "Gas–liquid chromatography", referring to the mobile and stationary phases, respectively). Secondly, the column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled, whereas column chromatography (typically) has no such temperature control. Thirdly, the concentration of a compound in the gas phase is solely a function of the vapor pressure of the gas.

Gas chromatography is also similar to fractional distillation, since both processes separate the components of a mixture primarily based on boiling point (or vapor pressure) differences. However, fractional distillation is typically used to separate components of a mixture on a large scale, whereas GC can be used on a much smaller scale (i.e. microscale).

Gas chromatography is also sometimes known as vapor-phase chromatography (VPC), or gas–liquid partition chromatography (GLPC). These alternative names, as well as their respective abbreviations, are

frequently used in scientific literature. Strictly speaking, GLPC is the most correct terminology, and is thus preferred by many authors.[30]

Gas chromatography–mass spectrometry (GC-MS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples. GC-MS can also be used in airport security to detect substances in luggage or on human beings. Additionally, it can identify trace elements in materials that were previously thought to have disintegrated beyond identification.

GC-MS has been widely heralded as a "gold standard" for forensic substance identification because it is used to perform a specific test. A specific test positively identifies the actual presence of a particular substance in a given sample. A non-specific test merely indicates that a substance falls into a category of substances. Although a non-specific test could statistically suggest the identity of the substance that could lead to false positive identification.[31]

Radioimmunoassay (RIA) is a very sensitive in vitro assay technique used to measure concentrations of antigens (for example, hormone levels in the blood) by use of antibodies. As such, it can be seen as the inverse of a radio binding assay, which quantifies an antibody by use of corresponding antigens.

Although the RIA technique is extremely sensitive and extremely specific, requiring specialized equipment, it remains the least expensive method to perform such tests. It requires special precautions and licensing, since radioactive substances are used. Today it has been supplemented by the ELISA method, where the antigen-antibody reaction is measured using calorimetric signals instead of a radioactive signal. However, because of its robustness, consistent results and low price per test, RIA methods are again becoming popular. It is generally simpler to perform than a bioassay.

The RAST test (radioallergosorbent test) is an example of radioimmunoassay. It is used to detect the causative allergen for an allergy.[32]

Haley et al (1983)[33] conducted a study in which, Biochemical determinations of plasma and salivary cotinine and thiocyanate were used to differentiate smokers from non-smokers and to follow daily smoking patterns in smokers. Results indicate that cotinine is better suited than thiocyanate to determine smoking status in large scale epidemiologic studies and to follow alterations in smoking behavior over periods of time. Salivary cotinine is a reliable alternative to plasma for validation of smoking status and for following changes in daily smoking patterns.

Jarvis et al (1984)[34] conducted a study in which, One hundred non-smoking patients attending hospital outpatient clinics reported their degree of passive exposure to tobacco smoke over the preceding three days and provided samples of blood, expired air, saliva and urine. Although the absolute levels

were low, the concentration of cotinine in all body compartments surveyed was systematically related to self-reported exposure. Salivary nicotine concentration also showed a linear increase with degree of reported exposure, although this measure was sensitive only to exposure on the day of testing. Measures of carbon monoxide, thiocyanate and plasma nicotine concentrations were unrelated to exposure. The data indicate that cotinine provides a valid marker of the dose received from passive smoke exposure. The non-invasive samples of urine and saliva are particularly suited to epidemiological investigations. Detailed questionnaire items may also give valuable information.

Machacek and Jiang et al (1986)[35] carried out a study in which, measurement of cotinine, a nicotine metabolite, has been studied as a method for monitoring smoking behavior and determining smoking status. We describe a specific, sensitive method for quantifying it in plasma and saliva by reversed-phase paired-ion liquid chromatography and detection by absorbance at 257 mm. The cotinine is extracted with methylene chloride and 2-phenylimidazole is the internal standard. Cotinine peak heights are linearly related to the amount on the column from 0 to 500 ng. The mean (± SD) concentration of cotinine in plasma of 31 passively exposed nonsmokers was 2.1±1.6p.g/L (range, 0-7.9 g/L). The regression of saliva cotinine concentration (y) on plasma cotinine concentration (x) at 0, 24, and 48 h in 10 smokers who refrained from smoking for 48 h was y (p.g/L) = 1.155x (g/L) +

0.245 (r =0.986). The efficacy of cotinine as a biological marker was determined at 0, 24, and 48 h of smoking abstinence.

Jarvis et al (1987)[24] conducted a study in which questionnaire and biochemical measures of smoking were studied in 211 hospital outpatients. Eleven different tests of smoke intake were compared for their ability to categorize smokers and nonsmokers correctly. The concentration of cotinine, whether measured in plasma, saliva, or urine, was the best indicator of smoking with sensitivity of 96-97 percent and specificity of 99-100 percent. Thiocyanate provided the poorest discrimination. Carbon monoxide measured as blood carboxyhaemoglobin or in expired air gave sensitivity and specificity of about 90 percent. Sensitivities of the tests were little affected by the presence among the claimed nonsmokers of a group of 21 "deceivers" who concealed their smoking. It is concluded that cotinine is the measure of choice, but for most clinical applications carbon monoxide provides an acceptable degree of discrimination and is considerably cheaper and simpler to apply.

Abrams et al (1987)[36] performed a study to determine the accuracy and reliability of saliva cotinine as an objective measure of smoking status was examined in two field studies. Saliva was collected from smokers and nonsmokers with repeated samples taken from a randomly selected subset of the smokers. Results indicated perfect classification of smokers versus nonsmokers and acceptable reliability of repeated samples. Using a cut-off of 10 ng/ml as suggested by Benowitz, perfect discrimination of smokers from

nonsmokers was achieved. All smokers had salivary cotinine levels greater than 10 ng/ml (mean = 349.2, SD = 195.4, range = 26-933) and all of the nonsmokers had levels of less than 10 ng/ml (mean = 0.3, SD = 1.6, range = 0-9).

Coutlas et al (1987)[37] conducted a population-based household survey of respiratory disease in 2,029 children and adults and measured salivary cotinine levels by radioimmunoassay in 1,360 nonsmokers and ex-smokers. At all ages median and mean cotinine levels among nonsmoker and ex-smokers increased with the number of smokers in the home. The prevalence of a detectable level of cotinine was about 35% for those not living with a cigarette smoker and was greater with the number of cigarettes smoked by household members. In a multiple logistic regression model, the major determinants of a detectable level of cotinine in children where mother's smoking (odds ratio (OR) » 3.2), father's smoking (OR = 2.1) and smoking of other household members (OR ~ 4.0). Among adults, the effects of spouse's smoking were smaller with OR = 1.3 and 1.4 for husband's and wife's smoking, respectively. They concluded that in the general population cotinine can be frequently detected in the saliva of nonsmokers, even among those not living with a smoker.

Langone et al (1988)[38] carried out a study to determine the value of a monoclonal antibody-based ELISA for measuring cotinine in saliva and urine of active and passive smokers was assessed. Cotinine (mean +/- SEM) was

detected in all 26 saliva (392 +/- 74 ng/ml) and 27 urine (4264 +/- 508 ng/ml creatinine; 2566 +/- 364 ng/ml) samples from smoking parents, but in only two of 36 saliva and one of 37 urines from nonsmokers (P less than 0.001). Similarly, mean cotinine levels in 30 saliva samples (4.67 +/- 1.10 ng/ml) and 33 urine samples (35.5 +/- 8.8 ng/mg creatinine; 25.3 +/- 8.1 ng/ml) from passively exposed children were significantly higher (P less than 0.001) than in fluids of 36 unexposed children. In adult smokers there was a positive correlation between salivary and urinary cotinine (P=0.002) and a close relationship between urinary cotinine and cigarettes smoked per day (P = 0.066). The ELISA gives a reliable quantitative measure of cotinine as an indicator of active and passive exposure to tobacco smoke. However, correlations with cotinine can be overestimated if large numbers of nonsmokers are included in the comparison.

Michael A wall et al (1988)[39] studied 98 subjects in age range of 24-66 years by gas liquid chromatography where the samples were categorized into non- smokers, passive and active smokers and found that only a minority of nonsmokers and detectable levels of cotinine in their serum (n=1) or saliva (n=1). The study concluded that active smokers of < 10 cigarettes per day had lower mean cotinine levels in both serum and saliva when compared to subjects who smoked >10 cigarettes.

Lee et al (1993)[40] conducted a study in which the current and passive smoking are associated with risk factors and the potential for confounding arising from these associations was studied using a representative sample of 9003 British adults. The distribution of 33 lifestyle factors generally considered associated with adverse health were compared in current smokers, ex-smokers, never smokers living with a smoker ("passive smokers") and never smokers not living with smokers of the 33 risk factors 27 showed a significantly higher prevalence in heavy smokers than in never smokers and only two showed a lower prevalence. For many risk factors, prevalence increased with amount smoked, decreased with time of smoking cessation and was increased in passive smokers. The possible magnitude of bias from confounding by the risk factors is estimated. It is concluded that confounding by multiple risk factors may be an important issue in smoking studies where weak associations are observed. This applies particularly to studies investigating the possible association of passive smoking with various health effects.

Istvan et al (1994)[41] carried out a study which investigates the relation of salivary cotinine and of the reported number of cigarettes smoked per day to body mass index among middle-aged male (n = 3,538) and female (n = 2,096) cigarette smokers participating in screening for entry to a clinical trial of early intervention in chronic obstructive pulmonary disease (Lung Health Study) from 1986 to 1989. Both before and after controlling for age,

education, and alcohol intake, the number of cigarettes smoked per day was positively related to body mass index among both men and women, whereas salivary cotinine levels were negatively related to body mass index among both men and women. The opposite relation of salivary cotinine and of reported number of cigarettes smoked per day to body mass index is discussed with regard to nicotine metabolism, energy intake, and measurement issues in the assessment of cigarette smoke exposure.

Ettar et al (2000)[42] collected self-reported data on smoking habits and saliva samples that were analyzed for cotinine concentration in 222 smokers and 97 nonsmokers. Participants were members of the University of Geneva (Switzerland) in 1995. The 207 cigarette-only smokers smoked on average 10.7 cigarettes/day and had a median concentration of cotinine of 113 ng/ml. The cotinine concentration was moderately associated with the number of cigarettes smoked per day (+14 ng/ml per additional cigarette, $p < 0.001$, Ff = 0.45) and was 54 ng/ml higher in men than in women after adjustment for cigarettes per day and for the Fagerstrom test for Nicotine Dependence. The cotinine level was not associated with the nicotine yield of cigarettes (r= 0.08). In nonsmokers, the median concentration of cotinine was 2.4 ng/ml. The cotinine concentration was 1.5 times higher in nonsmokers whose close friends/spouses were smokers than in nonsmokers ($p = 0.05$). A cutoff of 7 ng/ml of cotinine distinguished smokers from nonsmokers with a sensitivity of 92.3% and a specificity of 89.7%; a cutoff of 13 ng/ml provided equally

satisfactory results (sensitivity, 86.5%; specificity, 95.9%). This study provides evidence for the construct validity of both questionnaires and saliva cotinine for the assessment of active and passive exposure to tobacco smoke.

Nikajima et al (2000)[43] demonstrated, highly sensitive and reliable method for the determination of nicotine and its metabolite cotinine in human plasma by high-performance liquid chromatography was developed. Nicotine and cotinine were extracted from alkalinized plasma with dichloromethane and the volatility of nicotine was prevented by the addition of conc. HCl to the organic solvent during evaporation. The sensitivity of quantification at 260 nm absorption was improved by using a noise-base clean Uni-3 to 0.2ng/ml nicotine and 1.0 ng/ml cotinine. The method was validated over linear ranges of 0.2–25.0 ng/ml for nicotine and 1.0–80.0 ng/ml for cotinine.

TOPIC OF STUDY:

Estimation and comparison of serum and saliva cotinine level in smokers and non- smoker.

Study design: The Present study is an analytical case control study

Study duration: This study was conducted between March 2012 to August 2012 in the Department of Oral Medicine and Radiology of Ragas Dental College and Hospital with laboratory support from Sri Ramachandra Medical college, Porur, Chennai.

Study population:

A total number of 30 patients (15 smokers and 15 non- smokers) were involved in the study.

Obtaining approval from the authorities:

Permission from the Institutional Review Board of Ragas Dental College & Hospital, Chennai was obtained before starting the study.

Consent letter from the participants of the study was obtained in both Tamil and English.

MATERIALS

ARMAMENTARIUM USED

Examination of the patient

1. Dental chair with halogen lamp
2. Plain mouth mirror
3. Dental probe
4. Mouth mask
5. Disposable latex gloves

Salivary sample collection

1. Disposable mouth mask
2. A pair of sterile gloves
3. Sterile plastic containers for collection of saliva
4. Refrigerator

Blood sample collection (for extracting the serum)

- Disposable 5 ml plastic syringe and 23 gauge needle
- Vacutainer coated with Ethylene diamine tetra acetic acid (EDTA)
- Torniquet
- Sterile Cotton
- 70% alcohol as surface disinfectant

- Sterile vials
- Refrigerator

Cotinine estimation

1. Nicotinine and cotinine
2. Acetanilide
3. Sodium hydroxide
4. Dichloromethane
5. Conc. Hydrochloric acid
6. Vaccum evaporator
7. High performance liquid chromatography
    1. L – 7100 pumo
    2. L – 7400 u – v detector
    3. L – 7500 autosampler
    4. L – 7500 integrator
    5. 865 co- colum oven
    6. Phosphoric acid

METHODOLOGY

The study comprised of a total number of 30 male patients. Out of the 30 patients, 15 were non smokers and the other 15 were smokers

STUDY GROUP

The study group comprised of 15 male patients in the age group of 18 yrs and above with cigarrete smoking habit visiting the outpatient department of Ragas Dental College and Hospital, Chennai.

INCLUSION CRITERIA

- Male patients aged 18 yrs and above.
- Cigarrete smokers only

EXCLUSION CRITERIA

- Female patients
- Patients with Diabetes , hypertension and any known systemic diseases
- Patients who are currently under medication
- Other types of tobacco smokers

CONTROL GROUP

The control group comprised of 15 male patients in the age group of 18 yrs and above with no smoking habit visiting the outpatient department of Ragas Dental College and Hospital, Chennai.

INCLUSION CRITERIA

- Male patients aged 18 yrs and above.
- Non smokers.

EXCLUSION CRITERIA

- Female patients.
- Patients with Diabetes, hypertension and any known systemic diseases.
- Patients who are currently under medication.

INFORMED CONSENT

Permission from Institutional Review Board of Ragas Dental College and Hospital, Uthandi and Innovis Laboratory, Sri Ramachandra Medical College and Research institute, Porur, Chennai was obtained before starting the study.

Informed consent was obtained from all the subjects before including them in the study. Consent was prepared in both Tamil and English in letter format.

EXAMINATION OF THE SUBJECTS

The patients included in the study were made to sit comfortably on a dental chair and interrogated for demographic details and habits. An intraoral

examination was carried under halogen light. The findings were recorded in the proforma. Serum and saliva were collected.

SERUM SAMPLE COLLECTION[42]

1. Blood samples are taken from the vein in the antecubital fossa. The tourniquet is set around the upper arm of the subject, search for the cubital vein by inspecting and palpating and then sterilize the injection site. The vein can be anchored by placing the thumb about two centimeters below the vein and pulling gently to make the skin a little taut. After that, the needle, beveled upward, should be pushed smoothly and quickly into the vein, to minimize the possibility of hemolysis as a result of vascular damage. Immediately after the insertion, the tourniquet should be released to minimize the effect of hemoconcentration. 5 ml of venous blood was drawn. EDTA and Sodium Fluoride were added to prevent the coagulation of blood.
2. For the determination of the nicotine concentration, the plasma sample (1 ml) was alkalinized by 50 ml of 10 M NaOH. After the addition of 10 ng of acetanilide as an internal standard, the mixture was extracted with 4 ml of dichloromethane by shaking for 10 min.
3. After centrifugation at 1000 g for 10min, 25 ml of conc. HCI added to the organic fraction for the determination of the nicotine concentration. The organic fraction was evaporated with a vacuum

evaporator at 408°c. The residue was redissolved in 100 ml of the mobile phase and then an 80-ml portion of the sample was subjected to HPIC

4. For the determination of the cotinine concentration, the plasma sample (0.5ml) was alkalinized by 25 ml of 10 M NaOH and extracted with 4 ml of dichloromethane by shaking for 10 min. the organic fraction was evaporated with a vacuum evaporator at 408c without the addition of conc. HCI

The residue was redissolved in 100ml of the mobile phase and then an 80ml portion of the sample was subjected to HPLC.

SALIVA SAMPLE COLLECTION

The subjects were required to abstain from drinking, smoking or using oral hygiene products for at least 1 hour before saliva collection. The patients were asked to rinse their mouth with water and were made to sit comfortably in a chair. The patients were asked to pool the saliva in the mouth till the fullness is felt and then asked to spit in the given sterile plastic container with 5 ml reading. This was repeatedly done for 5 times to collect 5 ml of saliva. The sample is freezed to - 20°c for the procedure to be carried out. All samples were centrifuged at 3000 rpm for 10 min to remove particulate materials and the clean supernatant was processed immediately for estimation of cotinine which was then subjected to HPLC.

High – performance liquid chromatography[42]

1. Chromatography was performed using an L-7100 pump (Hitachi, Tokyo, Japan), an L-7400 UV detector (Hitachi), an L-7200 auto sampler (Hitachi), an L-7500integrator (Hitachi), and an 865-CO column oven (Jasco, Tokyo, Japan)
2. The flow-rate was1.0ml/min and the column temperature was 358°C. The eluent was monitored at 260nm with a noise-base clean Uni -3 (Union, Gumma, Japan)
3. For the determination of the nicotine concentration, the analytical column was a Hichrome 5C18 (15034.6 mm, 5mm) column (Tomsic, Tokyo, Japan) and the mobile phase was 7% CH OH, 2 mm Nah PO, 3240.2% phosphoric acid, and 1mM heptane sulfonate sodium. For the determination of the cotinine con-centration, the analytical column was a capecell Pak C UG 120 (2503.6 mm, 4mm) column (Shiseido, 18 Tokyo, Japan) and the mobile phase was 2% CH OH, 2 mm Nah PO, 0.1% phosphoric acid, 324 and 1 mm heptane sulfonate sodium.
4. Nicotine was quantified by comparison with the standard curves using the HPLC peak height ratios to acetanilide. Cotinine was quantified by comparing the HPLC peak heights to those of authentic standard.

The values were entered in the case sheet proforma and subjected to statistical analysis

STATISTICAL ANALYSIS

All the data were entered in Microsoft excel sheets. Statistical analysis was done using SPSS software.

$$\text{Mean } (X) = \frac{\sum X}{N}$$

Chi Square Test

$$X^2 = \frac{\text{sum of (observed frequency – expected frequency)}^2}{\text{Expected frequency}} = \frac{\sum (O-E)^2}{E}$$

Mean: defined as sum of values (X) divided by the number of values (N) and denoted by.

$P > 0.05$ = Difference is not significant

$P \leq 0.05$ = Difference is significant (S)

$P \leq 0.01$ = Difference is highly significant (S)

$P \leq 0.001$ = Difference is very highly significant (HS)

Fig.1: Armamentarium for clinical examination

Fig.2: Serum Collection

Fig.3: Ependorff Tubes for saliva collection

Fig.4: Blood Sample Collection

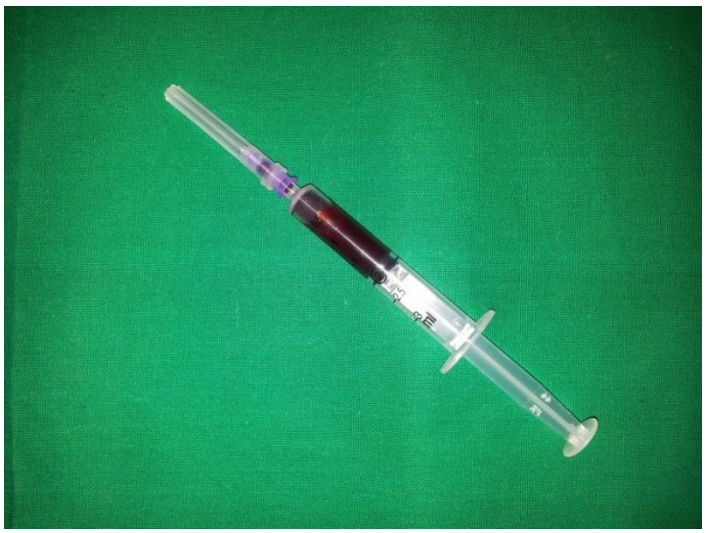


Fig.5: Blood sample

Fig.6: Saliva Sample Collection

Fig.7: Sample stored at – 20°C

Fig.8: Centrifuge Tubes

Fig.9: Lab Procedure Being Carried Out

Fig.10: Centrifuge Machine

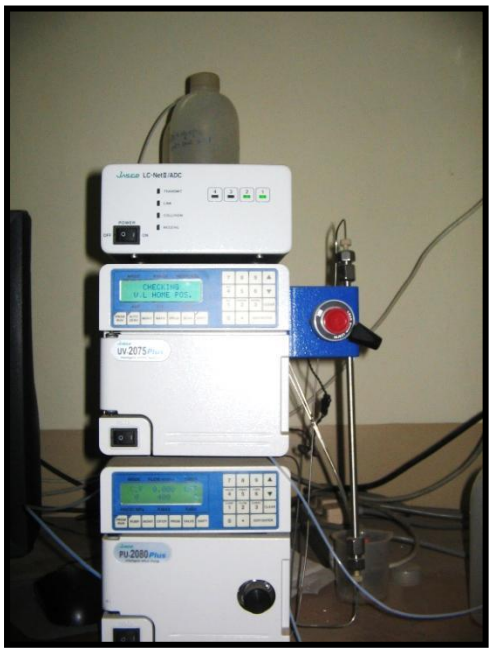


Fig.11: High Performance Liquid Chromatography System

The present study is a Case control study conducted by Ragas Dental College and Hospital and Sri Ramachandra Medical College and Research Institute, Porur, Chennai. It was devised to estimate and compare the Serum and Saliva cotinine levels in Smokers and Non smokers with High profile Liquid Chromatography. The study was conducted between March 2012 and August 2012 with15 Smokers and 15 non Smokers. The data obtained from the study was statistically analysed. The results extracted were compared with various variables included in the study and are presented here.

Table 1: Age wise distribution of Subjects in Control group

This table denotes age wise distribution of subjects in Control group. The age of 15 subjects in the control group was divided in to less than 30 and more than 30 years. Out of the 15 subjects 4 (26.7%) were in the age group of less than 30 years and 11 (73.3%) were in the age group of more than 30 years.

Table 2: Age wise distribution of Subjects in study group

This table denotes age wise distribution of subjects in study group. The age of 15 subjects in the study group was divided in to less than 30 and more than 30 years. Out of the 15 subjects 10 (66.7%) were in the age group of less than 30 years and 5 (33.3%) were in the age group of more than 30 years.

Table 3: Age wise distribution of Subjects in control and study groups

This table denotes age wise distribution of subjects in control and study group. Among the control group of 15 subjects, the mean age obtained is 34 years with a standard deviation of 19.13 and among the study group of 15 subjects the mean age obtained is 44 years with a standard deviation of 15.65. The p value obtained is 0.128 which is > 0.05 and is insignificant.

Table 4: Serum cotinine level in control group

This table denotes Serum cotinine level in control group. The Serum cotinine level in the control group was divided in to levels below 3 ng/dl and 3 – 5 ng/dl. Among 15 subjects Serum cotinine levels obtained were below 3 ng/dl in 12 (80%) subjects and between 3 – 5 ng/dl in 3 (20%) of the subjects.

Table 5: Serum cotinine level in study group

This table denotes Serum cotinine level in study group. The Serum cotinine level in the study group was divided in to levels below 5 - 50 ng/dl and above 50 ng/dl. Among 15 subjects Serum cotinine levels obtained were 5 - 50 ng/dl in 6 (40%) subjects and above 50 ng/dl in 9 (60%) of the subjects.

Table 6: Serum cotinine level in control and study groups

This table denotes Serum cotinine level in control and study group. Among 15 subjects in 12 (80%) of the control group subjects the Serum cotinine levels obtained were below 3 and 3 (20%) of the subjects showed

values in the range of 3- 5 ng/dl. In our study group subjects, the serum cotinine levels as well as saliva cotinine levels of below 3ng/dl and 3-5 ng/dl were found in zero subjects. Among 15 subjects in 12 (80%) of the study group subjects the Serum cotinine levels obtained were 5 – 50 ng/dl in 6 (40%) subjects and above 50ng/dl in 9 (60%) of the subjects. And none (0%) of the control group subjects showed values above 50 ng/dl and between 5-50 ng/dl. The p value obtained is 0.000 which is < 0.001 and is highly significant.

Table 7: Saliva cotinine level in control group

This table denotes saliva cotinine level in control group. The saliva cotinine level in the control group was divided in to levels below 3 ng/dl and 3–5 ng/dl. Among 15 subjects saliva cotinine levels obtained were below 3 ng/dl in 13 (86.7%) subjects and between 3–5 ng/dl in 2 (13.3%) of the subjects.

Table 8: Saliva cotinine level in study group

This table denotes saliva cotinine level in study group. The saliva cotinine level in the study group was divided in to levels between 5 - 50 ng/dl and above 50 ng/dl. Among 15 subjects saliva cotinine levels obtained were 5-50 ng/dl in 7 (46.7%) subjects and above 50 ng/dl in 8 (53.3%) of the subjects.

Table 9: Saliva cotinine level in control and study groups

This table denotes saliva cotinine level in control and study group. Among 15 subjects in 13 (86.7%) of the control group subjects the Serum saliva levels obtained were below 3 ng/dl and 2 (13.3%) of the subjects showed values in the range of 3- 5 ng/dl. And none (0%) of the study group subjects showed values below 3 ng/dl and between 3 – 5 ng/dl. Among 15 subjects in 7(46.7%) of the study group subjects the saliva cotinine levels obtained were 5 – 50 ng/dl and above 50 ng/dl in 8 (53.3%) of the subjects. And none (0%) of the control group subjects showed values 5 - 50 ng/dl and above 50 ng/dl. The p value obtained is 0.000 which is < 0.05 and is highly significant.

Table 10: Distribution of study group subjects according to number of cigarettes smoked per day

This table denotes the distribution of study group subjects according to number of cigarettes smoked per day. The number of cigarettes smoked per day was divided in to 1 – 10 and 11 – 30. Among 15 study group subjects 10 (66.7%) of them consumed 1 – 10 cigarettes per day and 5 (33.3%) of them consumed 11 – 30 per day.

Table 11: Distribution of study group subjects according to number of years of smoking

This table denotes the distribution of study group subjects according to number of years of smoking. The number of years of smoking was divided in to below 10 and 11 – 40 years. Among 15 study group subjects 12 (80%) of them were smoking for less than 10 years and 3 (20%) of them smoked in the range of 11 – 40 years.

Table 12: Correlation between age in year and Serum cotinine level in control group

This table denotes the correlation between age in year and Serum level in control group. Among 15 subjects all of the 4 (26.7%) subjects who were less than 30 years old showed values below 3 and among out of 11 (73.3%) of the subjects who were more than 30 years old 8 (72.7%) of them showed below 3 and 3(27.3%) of them showed values between 3-5. The p value obtained is 0.243 which is > 0.05 and is insignificant.

Table 13: Correlation between age in year and saliva cotinine level in control group

This table denotes the correlation between age in year and saliva level in control group. Among 15 subjects out of 4 (26.7%) subjects, 3 of them who were less than 30 years old showed values below 3 ng/dl and 1 subject showed value of 3 – 5 ng/dl. And out of 11 (73.3%) subjects who were more than 30

yrs. old, 10 of them showed values below 3 ng/dl and 1 subject between 3 – 5 ng/dl. The p value obtained is 0.423 which is > 0.05 and is insignificant.

Table 14: Correlation between Serum and saliva cotinine levels in control group

This table denotes correlation between Serum and saliva cotinine levels in control group. The Serum and saliva cotinine levels in control group was divided in to below 3 ng/dl and 3-5 ng/dl. Out of the 15 subjects 12 (80%) of them among which 11 showed values below 3 ng/dl and 1 showed value in the range of 3 – 5 ng/dl. Out of the 3(20%) of the subjects 2 of them showed values below 3 ng/dl and 1 showed between 3 – 5 ng/dl. The p value obtained is 0.255 which is > 0.05 and is insignificant.

Table 15: Correlation between Serum and saliva cotinine levels in study group

This table denotes correlation between Serum and saliva cotinine levels in study group. The Serum and saliva cotinine levels in study group was divided in to 5 – 50 ng/dl and above 50 ng/dl. Out of the 15 subjects 6 (40%) subjects out of which 5 showed values in the range of 5 – 50 ng/dl and 1 subject showed value above 50 ng/dl and 9 (60%) subjects out of which 2 of them showed values of 5 – 50 ng/dl and 7 showed above 50 ng/dl. The p value obtained was 0.020 which is < 0.05 and is significant.

Table 16: Correlation between age in year and Serum cotinine level in study group

This table denotes the correlation between age in year and Serum level in study group. The age group was divided in to less than 30 and more than 30 and Serum level was divided in to 5 – 50 ng/dl and above 50ng/dl. Among 15 subjects 10 (66.7%) of them who were less than 30 years old, out of which 5 of them showed values of 5 – 50 ng/dl and 5 of them showed values more than 50 ng/dl, and in 5 (33.3%) of the subjects who were more than 30 years old 1 subject showed value of 5-50 ng/dl and 4 showed values more than 50 ng/dl. The p value obtained is 0.264 which is > 0.05 and is insignificant.

Table 17: Correlation between age in year and saliva cotinine level in study group

This table denotes the correlation between age in year and saliva level in study group. The age group was divided in to less than 30 and more than 30 and saliva level was divided in to 5 – 50 ng/dl and above 50ng/dl. Among 15 subjects 10 (66.7%) of them who are less than 30 years old out of which 7 of them showed values 5 – 50 ng/dl and 3 of them showed values more than 50 ng/dl. And in 5 (33.3%) of the subjects none of them showed values 5–50 ng/dl and 5 of them showed values above 50 ng/dl. The p value obtained is 0.010 which is < 0.05 and is significant.

Table 18: Correlation between number of cigarettes smoked per day and serum cotinine level in study group

This table denotes correlation between number of cigarettes smoked per day and plasma level in study group. The number of cigarettes consumed was divided in to 1 – 10 and 11 – 30 and plasma level was divided in to 5 – 50 and above 50ng/dl. Among 15 subjects 10 (66.7%) of them were smoking in the range of 1-10 cigarettes per day out of which 6 of them show values in the range of 5 – 50 ng/dl and 4 of them showed value above 50 ng/dl and in those subjects who were smoking in the range of 11-30 cigarettes per day, all 5 (33.3%) of them showed values above 50 ng/dl. The p value obtained is 0.025 which is < 0.05 and is significant.

Table 19: Correlation between number of cigarettes smoked per day and saliva cotinine level in study group

This table denotes correlation between number of cigarettes smoked per day and saliva level in study group. The number of cigarettes consumed was divided in to 1 – 10 and 11 – 30 and saliva level was divided in to 5 – 50ng/dl and above 50ng/dl. Among 15 subjects 10 (66.7%) of them out of which 6 of them show values in the range of 5 – 50 ng/dl and 4 of them showed value above 50 ng/dl who were in the range of 1- 10 yrs. of smoking and in those subjects who were in 11-40 yrs. of smoking 5 (33.3%) of them among which 1 showed value of 5 – 50 ng/dl and 4 of them showed value

above 50 ng/dl. The p value obtained is 0.143 which is > 0.05 and is insignificant.

Table 20: Correlation between number of years of smoking and Serum cotinine level in study group

This table correlation between number of years of smoking and Serum cotinine level in study group. The number of years of smoking was divided in to below 10 and 11 – 40 years and Serum cotinine level was divided in to 5 – 50 ng/dl and above 50ng/dl. Among 15 subjects 12 (80%) of them out of which 5 of them showed values in the range of 5 – 50 ng/dl and 7 of them showed value above 50 ng/dl who were in the range of below 10 yrs. of smoking and in those subjects who are in 11-40 yrs. of smoking , 3 (20%) of them among which 1 showed value of 5 – 50 ng/dl and 2 of them showed value above 50 ng/dl. The p value obtained is 0.792 which is > 0.05 and is insignificant.

Table 21: Correlation between number of years of smoking and saliva cotinine level in study group

This table correlation between number of years of smoking and saliva cotinine level in study group. The number of years of smoking was divided in to below 10 and 11 – 40 years and saliva cotinine level was divided in to 5–50 ng/dl and above 50ng/dl. Among 15 subjects 12 (80%) of them out of which 7 of them showed values in the range of 5–50 ng/dl and 5 of them

showed value above 50 ng/dl who were in the range of ds below 10 yrs. of smoking and in those subjects who are in 11-40 yrs. of smoking all 3 of them showed value above 50 ng/dl and none of them showed values in range of 5- 50 ng/dl. The p value obtained is 0.070 which is > 0.05 and is insignificant.

Table 1: Age wise distribution of Subjects in Control group

Age	Frequency	Percent
Less than 30	4	26.7
More than 30	11	73.3
Total	15	100.0

Table 2: Age wise distribution of Subjects in study group

Age in years	Frequency	Percent
Less than 30	10	66.7
More than 30	5	33.3
Total	15	100.0

Table 3: Age wise distribution of Subjects in control and study groups

Age group in year	Number	Mean Age	Std. Deviation	P Value
Non Smoking	15	34.07	19.13	0.128
Smoking	15	44.07	15.65	

Table 4: Serum cotinine level in control group

Serum cotinine level in ng/dl	Frequency	Percent
Below 3	12	80.0
3-5	3	20.0
Total	15	100.0

Table 5: Serum cotinine level in study group

Serum Cotinine level in ng/dl	Frequency	Percent
5-50	6	40.0
Above 50	9	60.0
Total	15	100.0

Table 6: Serum cotinine level in control and study groups

Serum cotinine level in ng/dl		Group		Total	P Value
		Non-Smokers	Smokers		
Below 3	Count	12	0	12	0.000
	% within Serum cotinine level in ng/dl	100.0%	.0%	100.0%	
	% within Group	80.0%	.0%	40.0%	
3-5	Count	3	0	3	
	% within Serum cotinine level in ng/dl	100.0%	.0%	100.0%	
	% within Group	20.0%	.0%	10.0%	
5-50	Count	0	6	6	
	% within Serum cotinine level in ng/dl	.0%	100.0%	100.0%	
	% within Group	.0%	40.0%	20.0%	
Above 50	Count	0	9	9	
	% within Serum cotinine level in ng/dl	.0%	100.0%	100.0%	
	% within Group	.0%	60.0%	30.0%	
Total	Count	15	15	30	
	% within Serum cotinine level in ng/dl	50.0%	50.0%	100.0%	
	% within Group	100.0%	100.0%	100.0%	

Table 7: Saliva cotinine level in control group

Saliva cotinine level in ng/dl	Frequency	Percent
Below 3	13	86.7
3-5	2	13.3
Total	15	100.0

Table 8: Saliva cotinine level in study group

Saliva cotinine level in ng/dl	Frequency	Percent
5-50	7	46.7
Above 50	8	53.3
Total	15	100.0

Table 9: Saliva cotinine level in control and study groups

Saliva cotinine level in ng/dl		Group		Total	P. Value
		Non-Smokers	Smokers		
Below 3	Count	13	0	13	0.000
	% within Saliva cotinine level in ng/dl	100.0%	.0%	100.0%	
	% within Group	86.7%	.0%	43.3%	
3-5	Count	2	0	2	
	% within Saliva cotinine level in ng/dl	100.0%	.0%	100.0%	
	% within Group	13.3%	.0%	6.7%	
5-50	Count	0	7	7	
	% within Saliva cotinine level in ng/dl	.0%	100.0%	100.0%	
	% within Group	.0%	46.7%	23.3%	
Above 50	Count	0	8	8	
	% within Saliva cotinine level in ng/dl	.0%	100.0%	100.0%	
	% within Group	.0%	53.3%	26.7%	
Total	Count	15	15	30	
	% within Saliva cotinine level in ng/dl	50.0%	50.0%	100.0%	
	% within Group	100.0%	100.0%	100.0%	

Table 10: Distribution of study group subjects according to number of cigarettes smoked per day

No. of cig/day	Frequency	Percent
1-10	10	66.7
11-30	5	33.3
Total	15	100.0

Table 11: Distribution of study group subjects according to number of years of smoking

No. of yrs. of smoking	Frequency	Percent
Below 10	12	80.0
11-40	3	20.0
Total	15	100.0

Table 12: Correlation between age in year and Serum cotinine level in control group

Age in years		Serum cotinine level in ng/dl		Total	P. Value
		Below 3	3-5		
Less than 30	Count	4	0	4	0.243
	% within Age in years	100.0%	.0%	100.0%	
	% within Serum cotinine level in ng/dl	33.3%	.0%	26.7%	
More than 30	Count	8	3	11	
	% within Age in years	72.7%	27.3%	100.0%	
	% within Serum cotinine level in ng/dl	66.7%	100.0%	73.3%	
Total	Count	12	3	15	
	% within Age in years	80.0%	20.0%	100.0%	
	% within Serum cotinine level in ng/dl	100.0%	100.0%	100.0%	

Table 13: Correlation between age in year and saliva cotinine level in control group

Age in years		Saliva cotinine level in ng/dl		Total	P. Value
		Below 3	3-5		
Less than 30	Count	3	1	4	0.423
	% within Age in years	75.0%	25.0%	100.0%	
	% within Saliva cotinine level in ng/dl	23.1%	50.0%	26.7%	
More than 30	Count	10	1	11	
	% within Age in years	90.9%	9.1%	100.0%	
	% within Saliva cotinine level in ng/dl	76.9%	50.0%	73.3%	
Total	Count	13	2	15	
	% within Age in years	86.7%	13.3%	100.0%	
	% within Saliva cotinine level in ng/dl	100.0%	100.0%	100.0%	

Table 14: Correlation between Serum and saliva cotinine levels in control group

Serum cotinine level in ng/dl		Saliva cotinine level in ng/dl		Total	P. Value
		Below 3	3-5		
Below 3	Count	11	1	12	0.255
	% within Serum cotinine level in ng/dl	91.7%	8.3%	100.0%	
	% within Saliva cotinine level in ng/dl	84.6%	50.0%	80.0%	
3-5	Count	2	1	3	
	% within Serum cotinine level in ng/dl	66.7%	33.3%	100.0%	
	% within Saliva cotinine level in ng/dl	15.4%	50.0%	20.0%	
Total	Count	13	2	15	
	% within Serum cotinine level in ng/dl	86.7%	13.3%	100.0%	
	% within Saliva cotinine level in ng/dl	100.0%	100.0%	100.0%	

Table 15: Correlation between Serum and saliva cotinine levels in study group

Serum cotinine level in ng/dl		Saliva cotinine level in ng/dl		Total	P.Value
		5-50	Above 50		
5-50	Count	5	1	6	0.020
	% within Serum cotinine level in ng/dl	83.3%	16.7%	100.0%	
	% within Saliva cotinine level in ng/dl	71.4%	12.5%	40.0%	
Above 50	Count	2	7	9	
	% within Serum cotinine level in ng/dl	22.2%	77.8%	100.0%	
	% within Saliva cotinine level in ng/dl	28.6%	87.5%	60.0%	
Total	Count	7	8	15	
	% within serum cotinine level in ng/dl	46.7%	53.3%	100.0%	
	% within Saliva cotinine level in ng/dl	100.0%	100.0%	100.0%	

Table 16: Correlation between age in year and Serum cotinine level in study group

Age in years		Serum cotinine level in ng/dl		Total	P. Value
		5-50	Above 50		
Less than 30	Count	5	5	10	0.264
	% within Age in years	50.0%	50.0%	100.0%	
	% within Serum cotinine level in ng/dl	83.3%	55.6%	66.7%	
More than 30	Count	1	4	5	
	% within Age in years	20.0%	80.0%	100.0%	
	% within serum cotinine level in ng/dl	16.7%	44.4%	33.3%	
Total	Count	6	9	15	
	% within Age in years	40.0%	60.0%	100.0%	
	% within Serum cotinine level in ng/dl	100.0%	100.0%	100.0%	

Tables 17: Correlation between age in year and saliva cotinine level in study group

Age in years		Saliva cotinine level in ng/dl		Total	P. Value
		5-50	Above 50		
Less than 30	Count	7	3	10	0.010
	% within Age in years	70.0%	30.0%	100.0%	
	% within Saliva cotinine level in ng/dl	100.0%	37.5%	66.7%	
More than 30	Count	0	5	5	
	% within Age in years	.0%	100.0%	100.0%	
	% within Saliva cotinine level in ng/dl	.0%	62.5%	33.3%	
Total	Count	7	8	15	
	% within Age in years	46.7%	53.3%	100.0%	
	% within Saliva cotinine level in ng/dl	100.0%	100.0%	100.0%	

Table 18: Correlation between number of cigarettes Smoked per day and Serum cotinine level in study group

No . of cig/day		Serum cotinine level in ng/dl		Total	P.Value
		5-50	Above 50		
1-10	Count	6	4	10	0.025
	% within No . of cig/day	60.0%	40.0%	100.0%	
	% within serum cotinine level in ng/dl	100.0%	44.4%	66.7%	
11-30	Count	0	5	5	
	% within No . of cig/day	.0%	100.0%	100.0%	
	% within serum cotinine level in ng/dl	.0%	55.6%	33.3%	
Total	Count	6	9	15	
	% within No . of cig/day	40.0%	60.0%	100.0%	
	% within Serum cotinine level in ng/dl	100.0%	100.0%	100.0%	

Table 19: Correlation between number of cigarettes smoked per day and saliva cotinine level in study group

No . of cig/day		Saliva cotinine level in ng/dl		Total	P. Value
		5-50	Above 50		
1-10	Count	6	4	10	0.143
	% within No . of cig/day	60.0%	40.0%	100.0%	
	% within Saliva cotinine level in ng/dl	85.7%	50.0%	66.7%	
11-30	Count	1	4	5	
	% within No . of cig/day	20.0%	80.0%	100.0%	
	% within Saliva cotinine level in ng/dl	14.3%	50.0%	33.3%	
Total	Count	7	8	15	
	% within No . of cig/day	46.7%	53.3%	100.0%	
	% within Saliva cotinine level in ng/dl	100.0%	100.0%	100.0%	

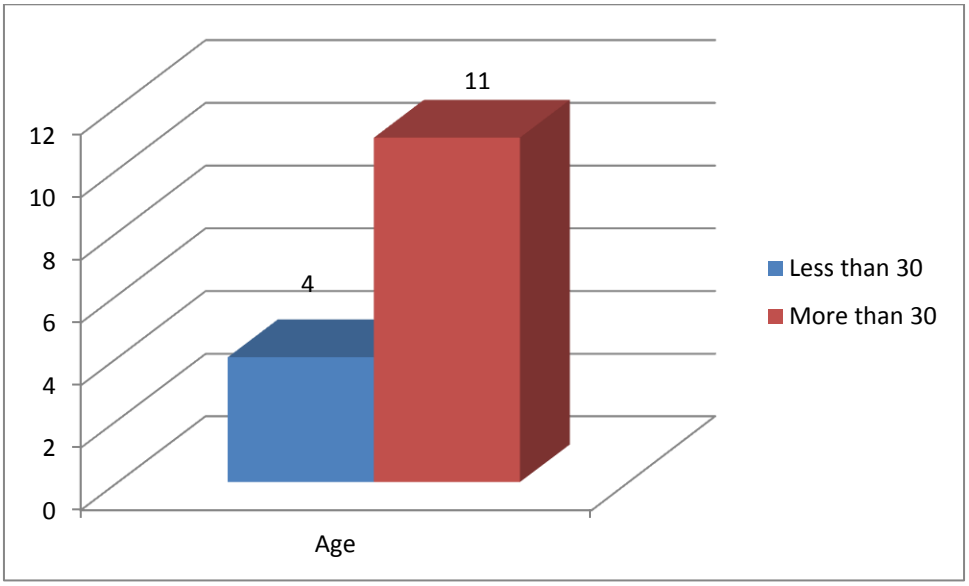
Table 20: Correlation between number of years of smoking and Serum cotinine level in study group

No. of yrs. of smoking		Serum cotinine level in ng/dl		Total	P. Value
		5-50	Above 50		
Below 10	Count	5	7	12	0.792
	% within No. of yrs. of smoking	41.7%	58.3%	100.0%	
	% within Serum cotinine level in ng/dl	83.3%	77.8%	80.0%	
11-40	Count	1	2	3	
	% within No. of yrs. of smoking	33.3%	66.7%	100.0%	
	% within Serum cotinine level in ng/dl	16.7%	22.2%	20.0%	
Total	Count	6	9	15	
	% within No. of yrs. of smoking	40.0%	60.0%	100.0%	
	% within serum cotinine level in ng/dl	100.0%	100.0%	100.0%	

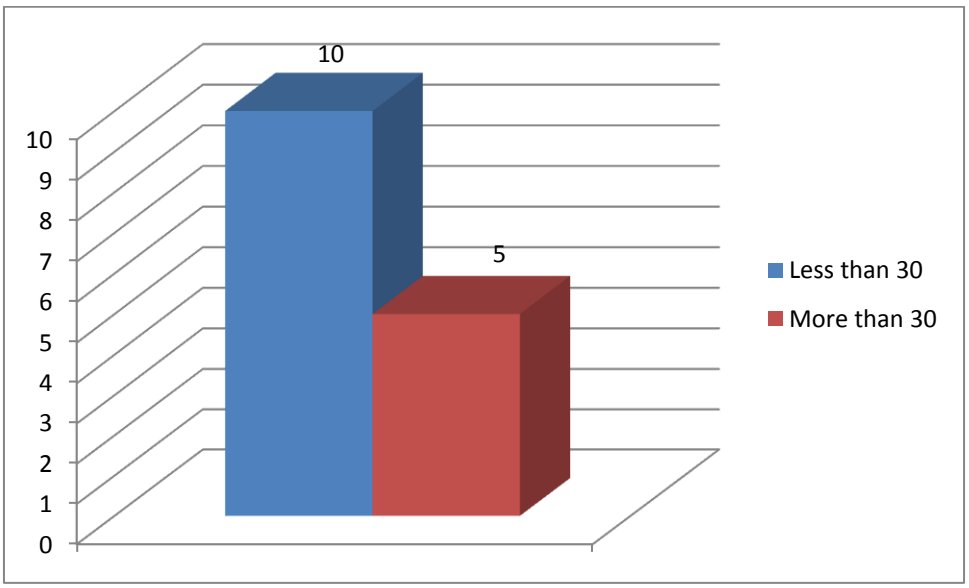
Table 21: Correlation between number of years of smoking and saliva cotinine level in study group

No. of yrs. of smoking		Saliva cotinine level in ng/dl		Total	P. Value
		5-50	Above 50		
Below 10	Count	7	5	12	0.070
	% within No. of yrs. of smoking	58.3%	41.7%	100.0%	
	% within Saliva cotinine level in ng/dl	100.0%	62.5%	80.0%	
11-40	Count	0	3	3	
	% within No. of yrs. of smoking	.0%	100.0%	100.0%	
	% within Saliva cotinine level in ng/dl	.0%	37.5%	20.0%	
Total	Count	7	8	15	
	% within No. of yrs. of smoking	46.7%	53.3%	100.0%	
	% within Saliva cotinine level in ng/dl	100.0%	100.0%	100.0%	

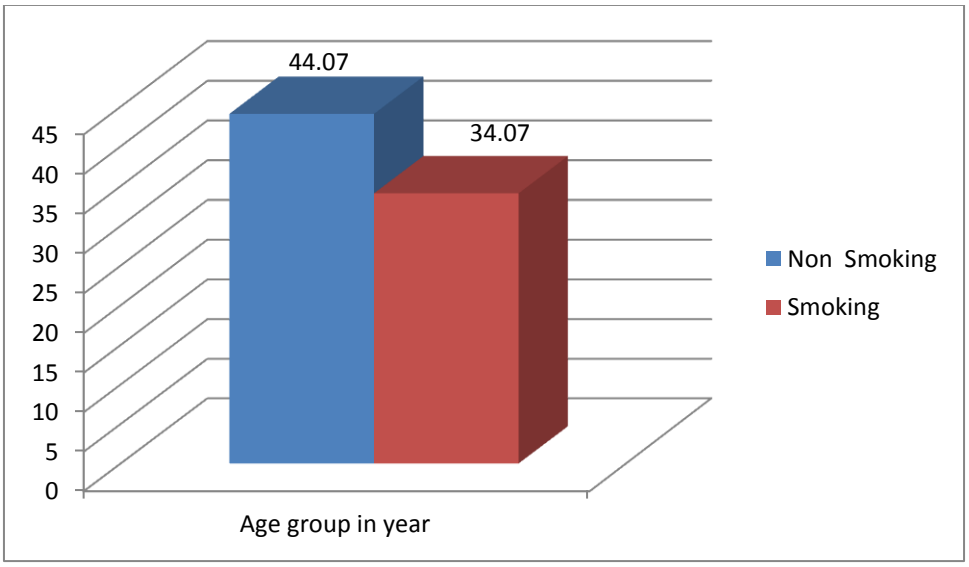
Grahp-1: Age wise distribution of Subjects in Control group



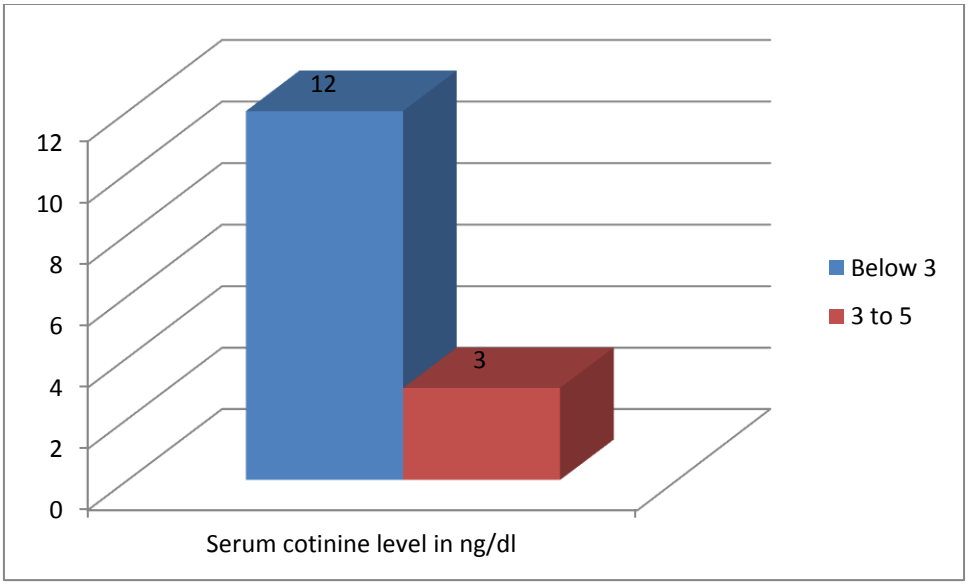
Graph-2: Age wise distribution of Subjects in study group



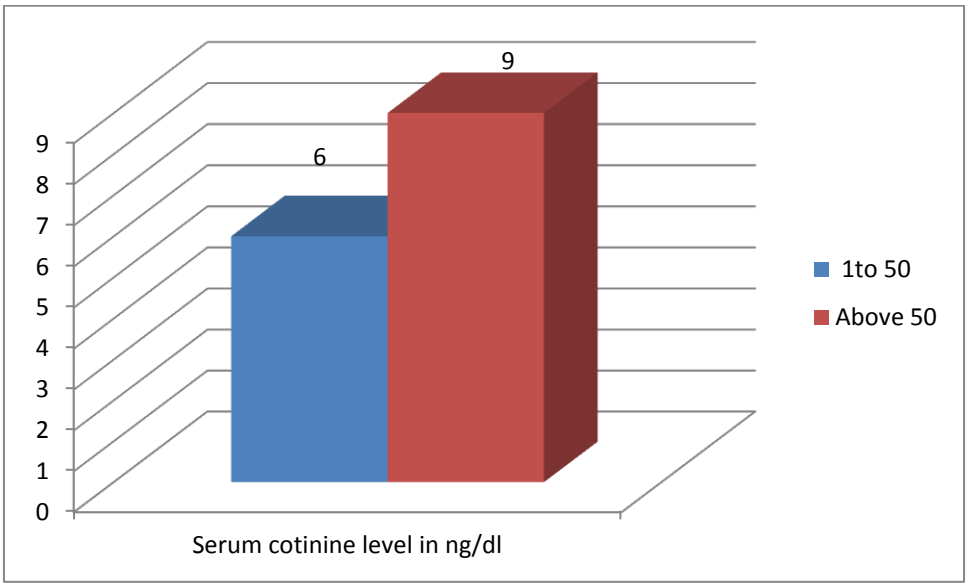
Graph-3: Age wise distribution of Subjects in control and study groups



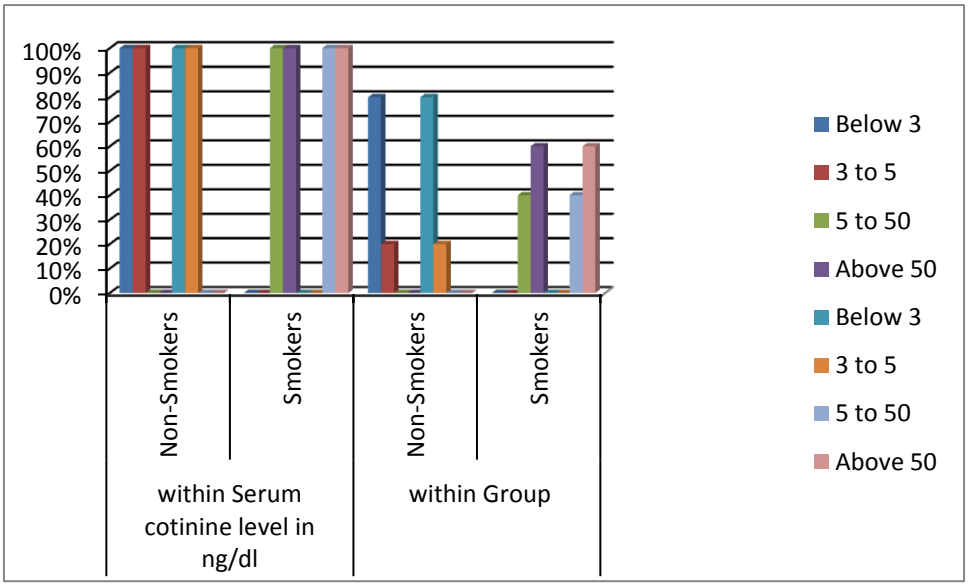
Graph-4: Serum cotinine level in control group



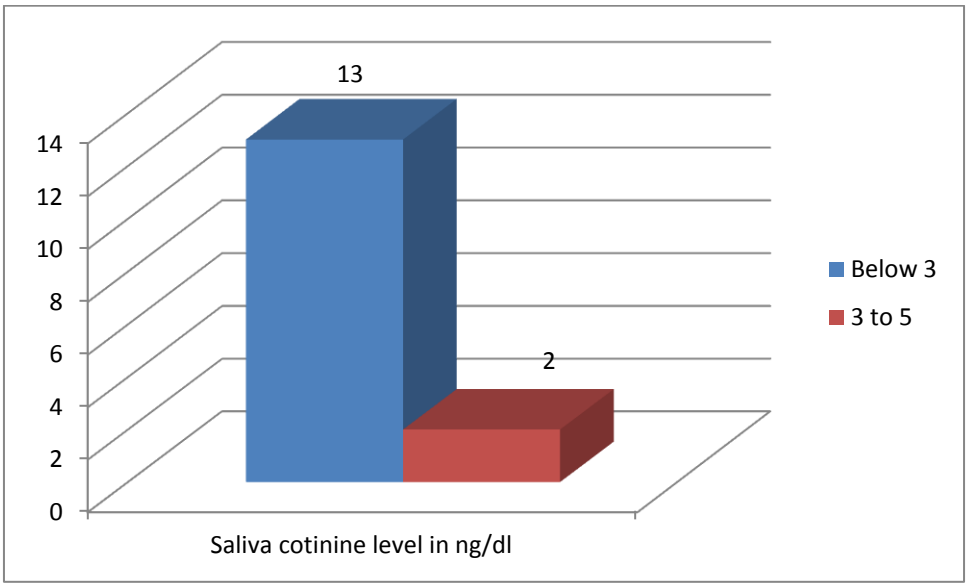
Graph-5: Serum cotinine level in study group



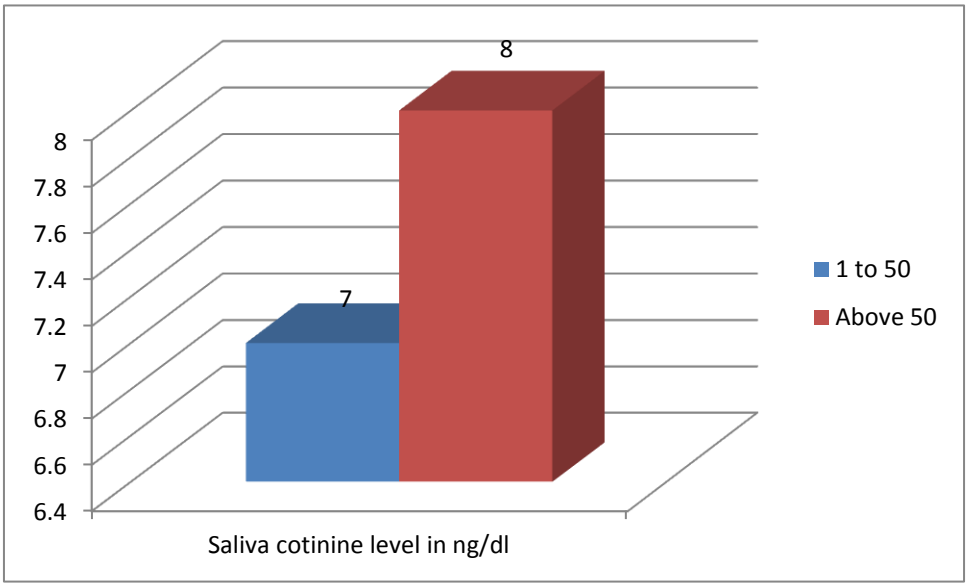
Graph-6: Serum cotinine level in control and study groups



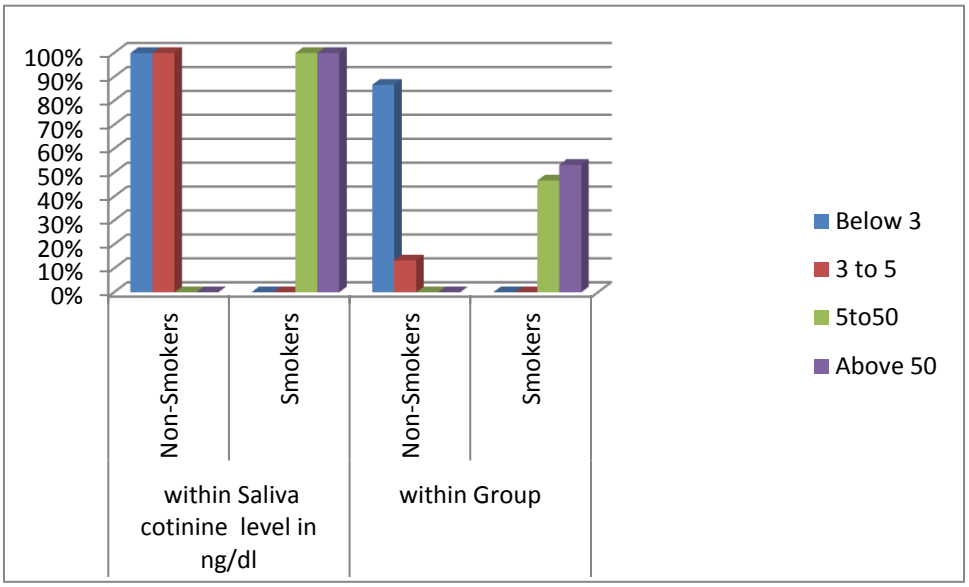
Graph-7: Saliva cotinine level in control group



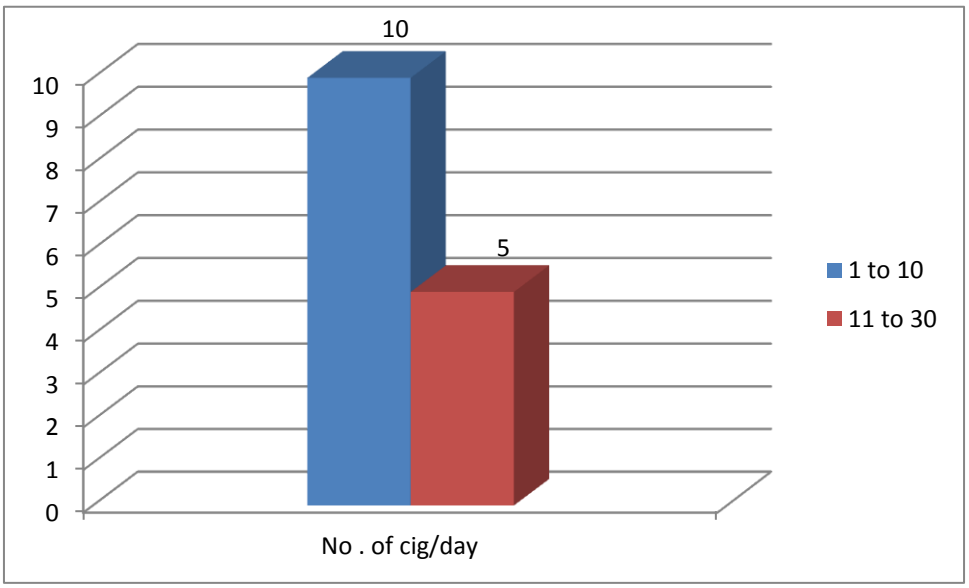
Graph-8: Saliva cotinine level in study group



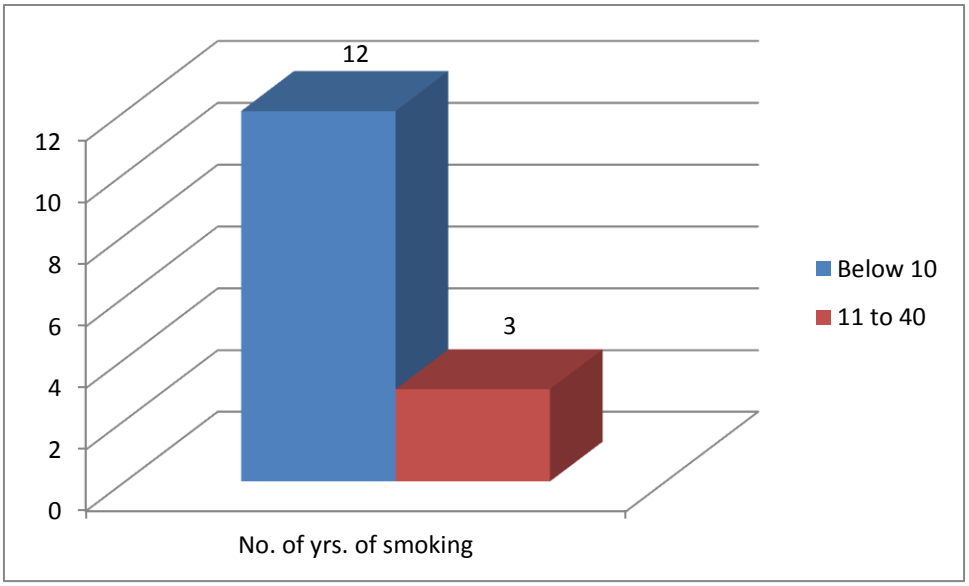
Graph-9: Saliva cotinine level in control and study groups



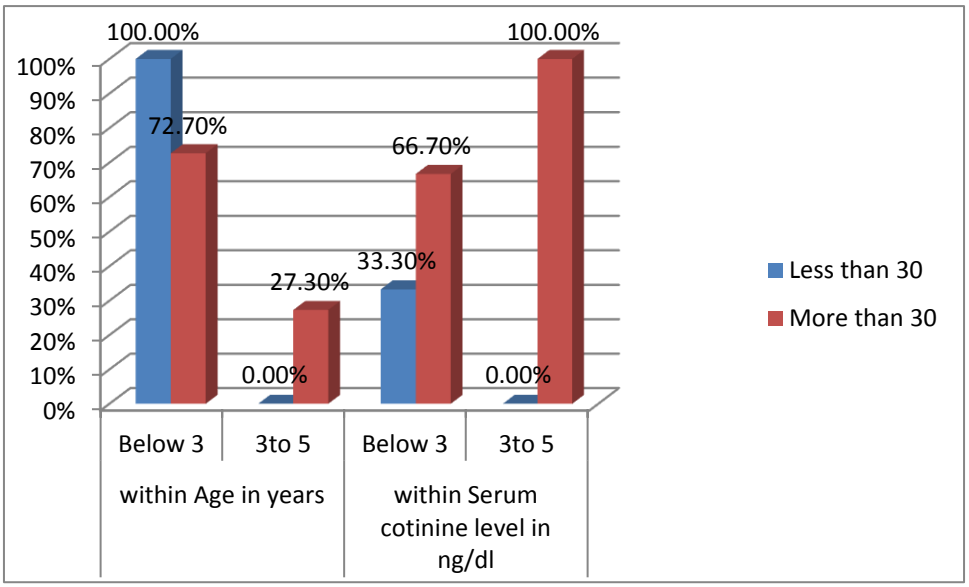
Graph-10: Distribution of study group subjects according to number of cigarettes smoked per day



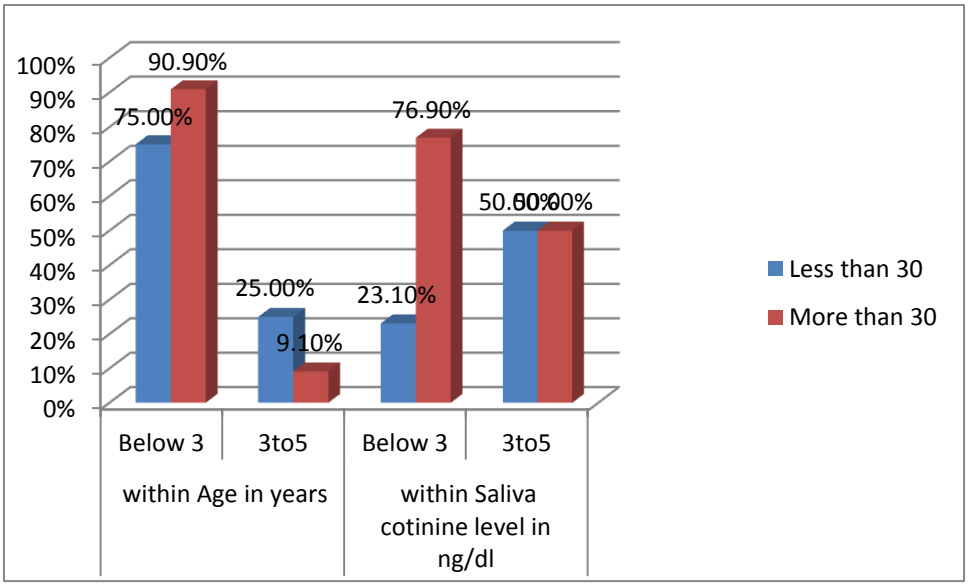
Graph-11: Distribution of study group subjects according to number of years of smoking



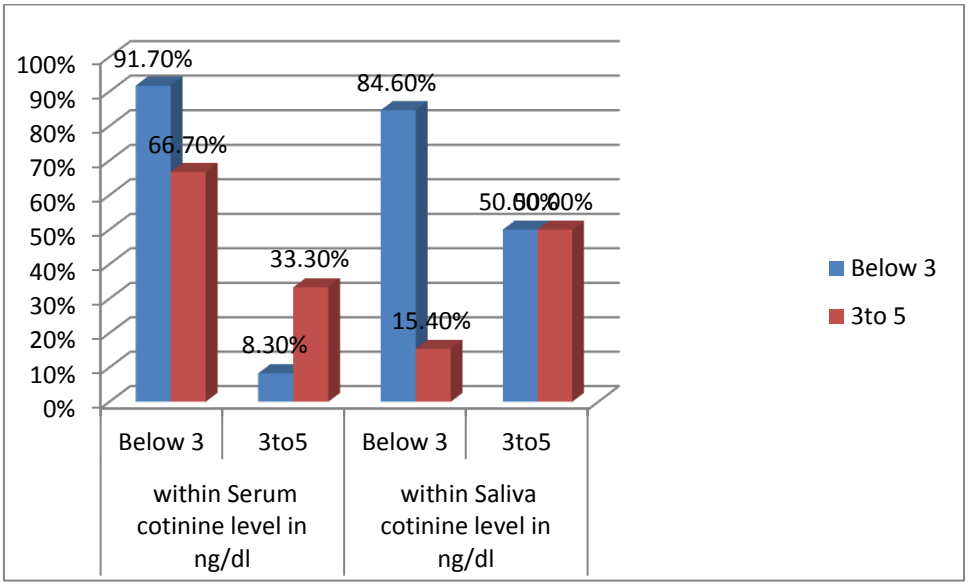
Graph-12: Correlation between age in year and Serum cotinine level in control group



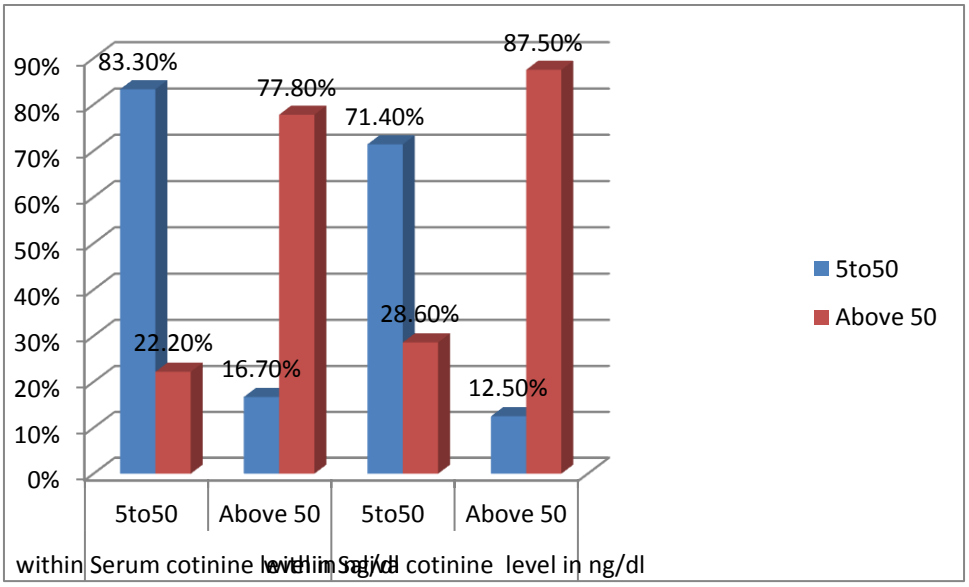
Graph-13: Correlation between age in year and saliva cotinine level in control group



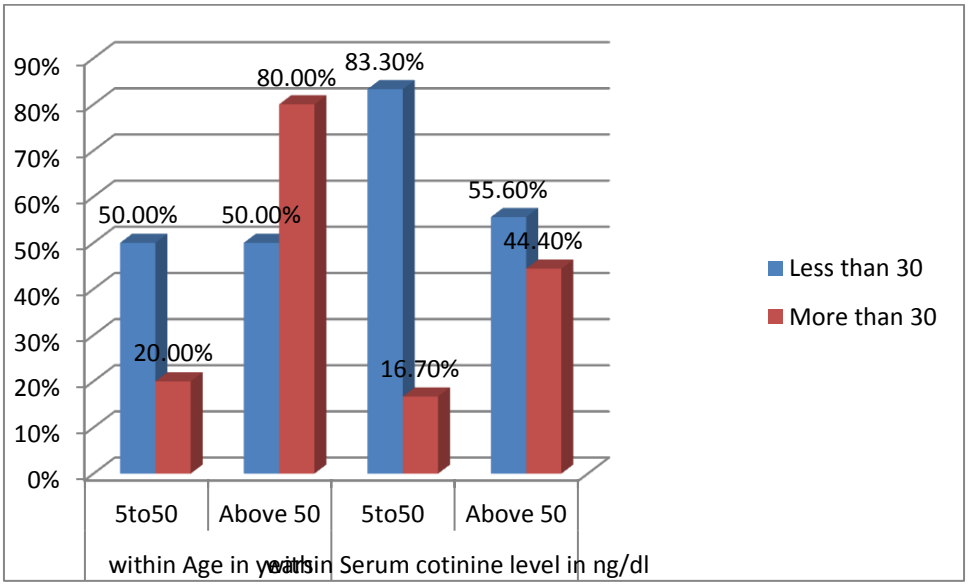
Graph-14: Correlation between Serum and saliva cotinine levels in control groups



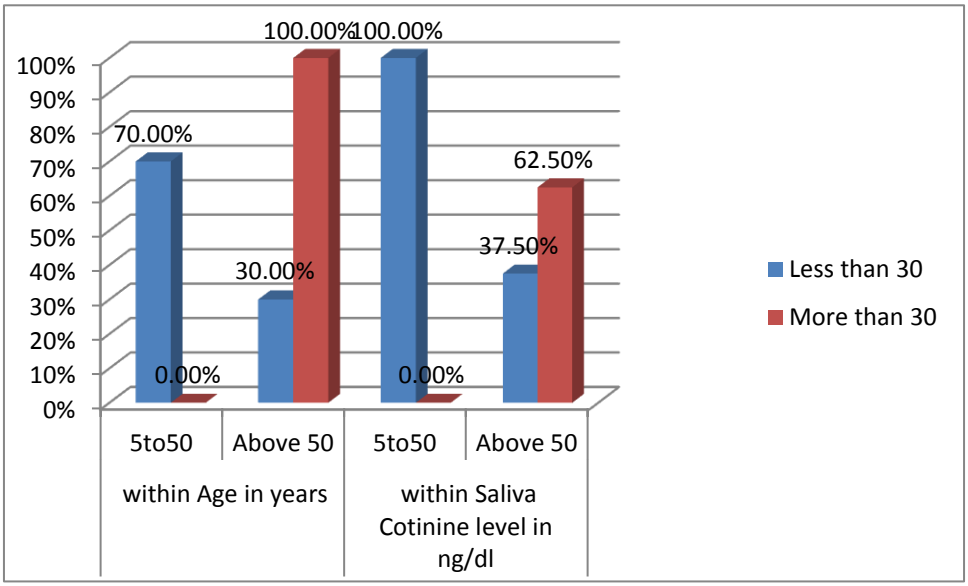
Graph-15: correlation between Serum and saliva cotinine levels in study group



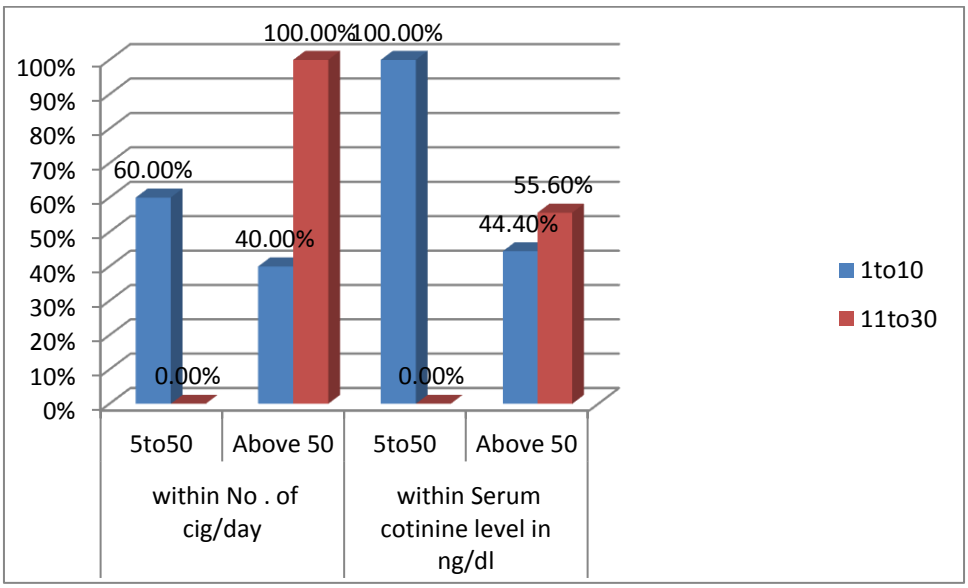
Graph-16: Correlation between age in year and Serum cotinine level in study group



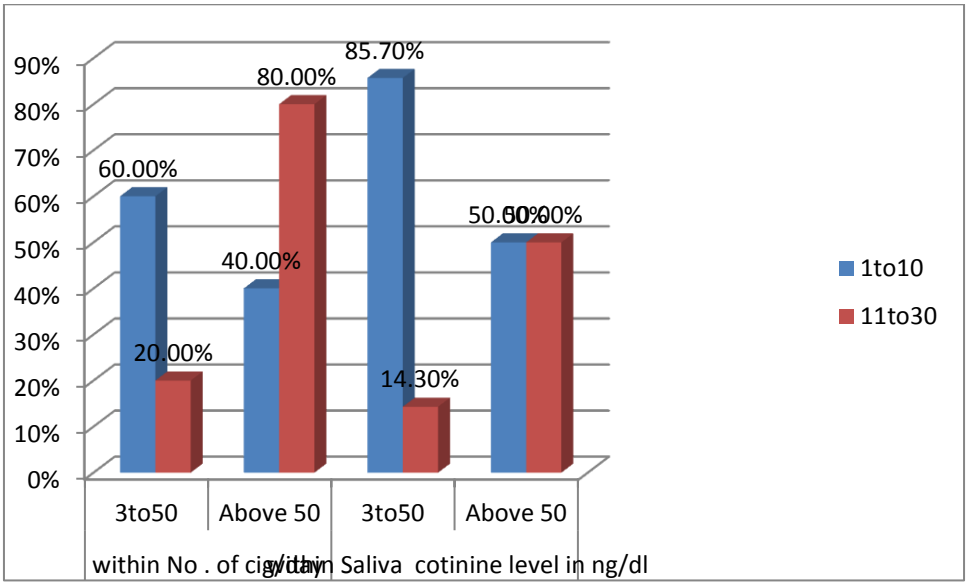
Graph-17: Correlation between age in year and saliva cotinine level in study group



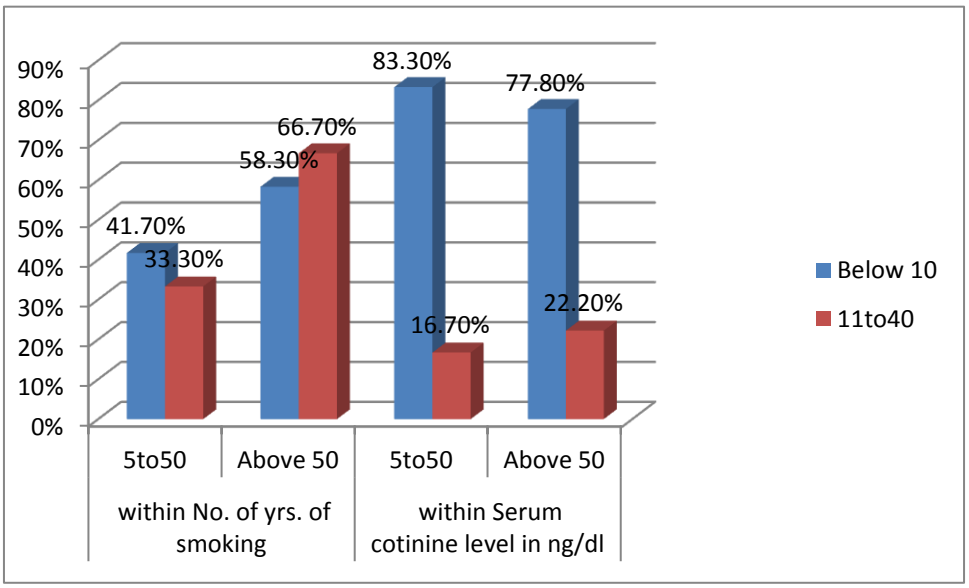
Graph-18: Correlation between number of cigarettes Smoked per day and Serum cotinine level in study group



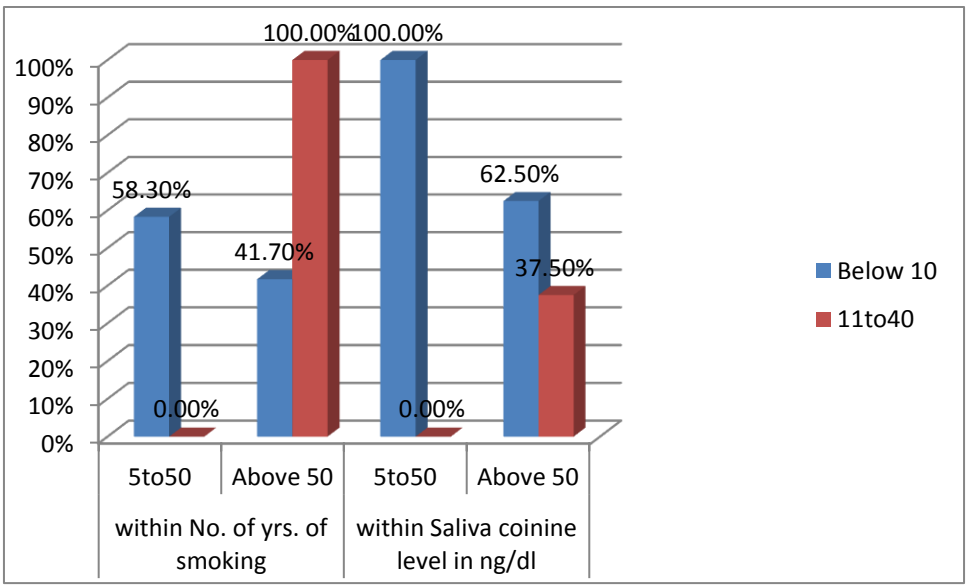
Graph-19: Correlation between number of cigarettes smoked per day and saliva cotinine level in study group



Graph-20: Correlation between number of years of smoking and Serum cotinine level in study group



Graph-21: Correlation between number of years of smoking and saliva cotinine level in study group



The World Health Organization predicts that tobacco deaths in India may exceed 1.5 million annually by 2020.[3]

Cigarette smoke is a complex mixture of components, such as carbon monoxide (CO), hydrogen cyanide (HCN) and nitrogen oxides, which are gases. Others, such as formaldehyde, acrolein, benzene and certain N-nitrosamines, are volatile chemicals contained in the liquid- vapor portion of the smoke aerosol. Still others, such as nicotine, phenol, polyaromatic hydrocarbons (PAHs), and certain tobacco-specific nitrosamines (TSNAs), are contained in the submicron-sized solid particles that are suspended in cigarette smoke.

In view of this chemical complexity, cigarette smoke has multiple, highly diverse effects on human health. It is not unexpected that multiple chemicals in cigarette smoke can contribute to any single adverse health effect.[10]

The adverse effects of smoking are not necessarily limited to active users of tobacco products. In recent years concern about health risks experienced by non smokers who are involuntarily exposed to tobacco smoke through passive smoking. ETS is a similar, but not identical to mainstream smoke inhaled during active smoking and many of hazardous substances known to be present in mainstream smoke are also present in ETS. In fact because of difference in combustion and aging some of the substances are

actually prevalent in ETS than in mainstream smoke. Strong evidence indicates that ETS represents a serious and substantial public health problem.[44]

Cotinine the major proximate metabolite of nicotine, has been widely used as a biomarker of exposure to tobacco and both active and second hand tobacco.[45]

The present study is a case control study conducted by Ragas Dental College and Hospital, Chennai and Sri Ramachandra Medical College, Porur, Chennai. It was devised to estimate and compare the serum and saliva cotinine levels in smokers and non-smokers with high profile liquid chromatography. The study was conducted between March 2012 and August 2012 with 15 smokers and 15 non-smokers.

Aim of the study is to estimate and compare cotinine level in smokers and Non- smokers in saliva and serum.

According to the most recent Government of India's National sample survey data, there are 184 million tobacco consumers in India. About 60% are tobacco smokers.

In our study, among the 30 male subjects where 15 of them are tobacco smokers, the age chosen was 18 years and above. Among the control group of 15 subjects 4 (26.7%) were in age group of less than 30 years and 11 (73.3%) were in age group of more than 30 years. This is in accordance with **Rani, Bonu, Jha, et al**[2] who studied prevalence and predictors of smoking in a

national cross sectional household survey of 315 598 individuals 15 years or older in National Family Health Survey-2 (1998–99).Hence the age of our control group have been matched in accordance with the studies mentioned.[2]

In our study among the 30 male subjects where 15 of them are tobacco smokers, the minimum age obtained was 20 years and maximum of 75 years. Among the study group of 15 subjects 10 (66.7%) subjects were in age group of less than 30 years and 5 (33.3%) subjects were in age group of more than 30 years. This is in accordance with **Rani, Bonu, Jha, et al[2]** who studied prevalence and predictors of smoking in a national cross sectional household survey of 315 598 individuals 15 years or older in National Family Health Survey-2 (1998–99).Their findings show that thirty per cent of the population 15 years or older smoke cigarrete, Their study also revealed that compared to the younger population (15–24 years), the older population (25+years) had a greater likelihood of smoking tobacco. The prevalence of tobacco consumption increased up to the age of 50 years.

In our control group of 15 subjects we obtained serum cotinine levels values between 0 and 5ng/dl. Serum cotinine levels obtained were below 3 in 12 (80%) subjects and between 3-5 in 3 (20%) of the subjects. Our results are in accordance with study by **Jarvis et al[24]** who studied 211 non smokers and obtained mean values of 2.3ng/dl. The Serum cotinine level in the study group was divided in to levels below 5-50ng/dl and above 50ng/dl. Among 15 subjects Serum cotinine levels obtained were 5-50 in 6 (40%) subjects and

above 50 in 9 (60%) of the subjects. **Jarvis et al**[34] who studied 211 smokers and obtained mean values of 164 ng/dl.

Their study stated that the concentration of cotinine, whether measured in Serum. saliva.or urine, was the best indicator of smoking. with sensitivity of 96-97 per cent and specificity of 99-100 per cent. The results of their study concluded that the concentration of certain biochemical markers of smoke intake in the body can give a categorization of smoking status which is substantially more accurate than self report. Though their study was based on Gas chromatography method, the values obtained are similar to our study.

Machacek and Jiang et al[35] measured plasma cotinine levels in non-smokers by Reversed- phase paired ion liquid chromatography. The mean (±SD) concentration of cotinine in plasma of 31 nonsmokers, obtained was 2.1 ±1.6 µg/L (range, 0-7.9 g/L) which is higher than the values obtained in our study. The discrepancy in the values maybe due to the difference in the methodology.

In our study group of 15 subjects serum cotinine levels were grouped in to values between 5 – 50ng/dl and more than 50ng/dl. Serum cotinine levels obtained were 5 – 50ng/dl in 6 (40%) subjects and above 50ng/dl in 9 (60%) of the subjects.

Machacek and Jiang et al[35] measured plasma cotinine levels in smokers by Reversed- phase paired ion liquid chromatography. The mean

(±SD) concentration of cotinine in plasma of 10 smokers, obtained was 150µg/l. which is higher than the values obtained in our study. The discrepancy in the values maybe due to the difference in the methodology and the sample size.

Our serum cotinine levels are not in accordance with**, Jarvis et al 1987**[24] who studied 211 patients and obtained values of 330+/- 190ng/dl. The discrepancy in the values may be due to the population studied, methodology used and smaller sample size.

On comparison of Serum cotinine levels in control and study group of 30 subjects 15 subjects in 12 (80%) of the control group subjects the Serum cotinine levels obtained were below 3 and 3 (20%) of the subjects showed values in the range of 3- 5 ng/dl. And none (0%) of the study group subjects showed values below 3 and between 3 – 5. Among 15 subjects in 12 (80%) of the study group subjects the Serum cotinine levels obtained were 5 – 50 in 6 (40%) subjects and above 50 in 9 (60%) of the subjects. And none (0%) of the study group subjects showed values above 50 and between 3 – 5. The p value obtained is 0.000 which is < 0.001 and is highly significant.

Among our control group of 15 subjects who are non-smokers saliva cotinine levels obtained were 0 – 5 ng/dl. We obtained values below 3 in 13 (86.7%) subjects and between 3 – 5 ng/din 2 (13.3%) of the subjects. This value is in accordance with, **Jarvis et al 1987**[27] who studied 211 patients and obtained values of 1.7 ng/dl.

Our salivary cotinine levels in non smokers are also in accordance with **Coutlas et al**[37] who obtained values of 1.6 +/- 2.8 ng/dl by Radio-immuno assay method which is in accordance with our study. This comparison also indicates that the saliva cotinine levels in non-smokers are within the range mentioned above irrespective of the methodology used.

Our results are in accordance with study conducted by **Abrams et al**[36] who obtained value of 0.3+/- 1.6 ng/dl. Their study concluded that and all of the nonsmokers had levels of less than 10 ng/ml.

Our study revealed zero saliva cotinine values in five subjects out of 15 control group subjects with rest of subjects showing values within 5ng/dl. **Michael A Wall et al**[39] studied 19 subjects in age range of 24- 66 years by gas liquid chromatography where the samples were categorized into non–smokers, passive and active smokers and found only a minority of non- smokers had detectable cotinine levels in their serum (n=1) or saliva (n=1).This discrepancy in the results are due to the fact that our control group sample was not categorized into passive smokers and non-smokers. Moreover all samples in Michael A Wall study were taken in early morning before going to work and hence they would not have been exposed to environmental tobacco exposure whereas samples in our study were taken between mid - morning and afternoon.

Our values are also in accordance with **Ettar et al**[42] who analysed 97 nonsmokers for cotinine concentration in saliva and obtained a concentration of 2.4ng/dl.

In our study group of 15 subjects saliva cotinine levels obtained were grouped in the range of 5 – 50 ng/dland above 50 ng/dl. saliva cotinine levels obtained were 5 – 50ng/dl in 7 (46.7%) subjects and above 50 ng/dlin 8 (53.3%) of the subjects.

Coutlas et al[37] also obtained salivary Cotinine values of 296 +/- 208 ng/dl in smokers by Radio-immuno Assay method which is in accordance with our study. This comparison also indicates that the saliva cotinine levels in smokers are within the range mentioned above irrespective of the methodology used.

Our values are also in accordance with **Ettar et al**[42] who analysed 207 smokers for cotinine concentration in saliva and obtained a concentration of 113ng/dl.

Our results are not in accordance with study conducted by **Abrams et al**[36] who obtained value of 348 +/- 195.4 ng/dl. Variation in results may be due to methodology used and population studied.

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On comparison of saliva cotinine levels in control and study group of 15 subjects 13 (86.7%) of the control group subjects the plasma saliva levels obtained were below 3 and 2 (13.3%) of the subjects showed values in the range of 3- 5 ng/dl. And none (0%) of the study group subjects showed values below 3 and between 3 – 5. Among 15 subjects in 7(46.7%) of the study group subjects the saliva cotinine levels obtained were 5 – 50 and above 50 in 8 (53.3%) of the subjects. And none (0%) of the study group subjects showed values 5 - 50 and above 50. The p value obtained is 0.000 which is < 0.001 and is highly significant.

On distribution of 15 study group subjects according to number of cigarettes consumed per day 10 (66.7%) of them consumed 1 – 10 cigarettes per day and 5 (33.3%) of them consumed 11 – 30 per day. On distribution of 15 study group subjects according to number of years of smoking 12 (80%) of them were smoking for less than 10 years and 3 (20%) of them smoked in the range of 11 – 40 years.

In our study we have obtained insignificant p values of > 0.05 on correlating age with serum and saliva cotinine levels in control group and positive correlation with a significant p value of < 0.05 was obtained on correlating age and saliva cotinine level and number of cigarettes consumed per day and serum cotinine level in study group. We obtained insignificant p value of > 0.05 on correlating serum and saliva cotinine levels in control

group We have obtained positive correlation with a significant p value of < 0.05 on correlating Serum and saliva cotinine levels in study group.

The correlation between number of years of smoking and serum and salivary cotinine levels were insignificant with a p value of > 0.05. This can be attributed to smaller sample size, variation in time of sampling, assessment using single sample and variation in individual circadian rhythm.

Further studies with modifications in above parameters would contribute to better results which would help us to establish saliva as effective diagnostic tool in estimation of cotinine levels in smokers for further management.

The present study titled Estimation of cotinine level in the saliva, serum in smokers and non-smokers was conducted between March 2012 and August 2012 by Ragas Dental College and Hospital in Ramachandra Medical College and Research Institute, Chennai to estimate cotinine levels in saliva and serum of smokers and nonsmokers using high profile liquid chromatography method.

The study group comprised of a total number of 30 patients. Out of 30 subjects 15 were non smokers and 15 of them were smokers. Informed consent was taken from all subjects before including them in the study. Participants with systemic diseases and those who are currently not under any medication were excluded from the study.

For the determination of the cotinine concentration, the serum sample (0.5 ml) was alkalinized by 25 ml of 10 M NaOH and extracted with 4 ml of dichloromethane by shaking for 10 min. The organic fraction was evaporated with a vacuum evaporator at 408C without the addition of conc. HCl.

The residue was redissolved in 100 ml of the mobile phase and then an 80 ml portion of the sample was subjected to HPLC. High-performance liquid chromatography Chromatography was performed using an L-7100 pump (Hitachi, Tokyo, Japan), an L-7400 UV detector (Hitachi), an L-7200 autosampler (Hitachi), an L-7500 integrator (Hitachi), and an 865-CO column oven (Jasco, Tokyo, Japan). The flow-rate was 1.0 ml /min and the column

temperature was 358C. The eluent was monitored at 260 nm with a noise-base clean Uni-3 (Union, Gunma, Japan.

The study documents the following data

- Age chosen in our study was 18 years and above. Among the control group of 15 subjects, the mean age obtained is 34 years with a standard deviation of 19.13 and among the study group of 15 subjects the mean age obtained is 44 years with a standard deviation of 15.65.
- In control group of 15 subjects, serum cotinine levels obtained were below 3 in 12 (80%) subjects and between 3–5 in 3 (20%) of the subjects.
- In study group of 15 subjects, serum cotinine levels obtained were 5 - 50 in 6 (40%) subjects and above 50 in 9 (60%) of the subjects.
- In control group of 15 subjects, saliva cotinine levels obtained were below 3 in 13 (86.7%) subjects and between 3 – 5 in 2 (13.3%) of the subjects.
- In study group of 15 subjects, saliva cotinine levels obtained were 5 - 50 in 7 (46.7%) subjects and above 50 in 8 (53.3%) of the subjects.
- Comparison of serum and saliva cotinine levels in control and study groups was highly significant with a p value of < 0.001.
- Among 15 study group subjects, 10 (66.7%) of them consumed 1 – 10 cigarettes per day and 5 (33.3%) of them consumed 11 – 30 per day.

- Among 15 study group subjects, 12 (80%) of them were smoking for less than 10 years and 3 (20%) of them smoked in the range of 11 – 40 years.
- We obtained insignificant correlations between age and serum cotinine levels in both control and study group.
- We obtained insignificant correlation between age and saliva cotinine level in control group and positive correlations between age and saliva cotinine level in study group.
- We obtained insignificant correlation between serum and saliva cotinine levels in control group and significant correlation between serum and saliva cotinine levels in study group.
- We obtained significant correlation between number of cigarettes consumed per day and serum level in study group.
- We obtained insignificant correlation between number of cigarettes consumed per day and saliva cotinine level in study group.
- We obtained insignificant correlation between number of years of smoking and serum and saliva cotinine levels in study group.

To conclude our study shows highly significant difference between serum and saliva cotinine levels between smokers and non smokers. Our study shows that Measurement of cotinine levels can provide a sensitive estimate of tobacco smoke exposure.

Data available in literature using HPLC method alone are scarce to arrive at conclusive results. For the purpose of developing epidemiologic studies, comparative data on relative sensitivities of cotinine measurements in serum and saliva with bigger sample size and in different population are required.

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MASTER CHART

SMOKERS – STUDY GROUP

S.No.	Name	Age	Sex	No. of cig/day	No. of yrs. of smoking	plasma level in ng/dl	saliva level in ng/dl
1	Meeran	72	M	20	40	183.6	69.7
2	Xavier	24	M	15	7	127.1	41.9
3	Ranjith	24	M	6	4	50.6	58.2
4	Sekar	26	M	30	15	76.4	77.3
5	Aravind	24	M	10	5	175.4	39
6	Dayalan	27	M	20	4	115.5	69.4
7	Dheeraj	23	M	4	5	49.1	25.8
8	Abraham	23	M	5	4	43.9	27
9	Peter	24	M	3	4	24.78	40.14
10	Thambaiya	85	M	8	30	20.7	67.9
11	Arumugam	42	M	10	10	76.8	97.8
12	Muthukumar	24	M	5	4	35.1	6.5
13	Sundar	22	M	4	3	46.9	25.5
14	Selvamani	33	M	5	6	80	84.7
15	Gajendran	38	M	15	7	114.3	80.2
Non Smokers - Control Group							
1	Srinivasan	42	M			1.09	0.9
2	Parthiban	36	M			2.83	2.06
3	Joeraman	25	M			0.09	0.1
4	Pearlcid	24	M			2	4
5	Munirammaiya	55	M			3.83	1.6
6	Narasimman	63	M			0.06	1.02
7	Parthiban	35	M			0	0.1
8	Radhakrishnan	65	M			3.5	0.001
9	Rajendran	41	M			2.4	2.85
10	Devaraj	52	M			0	0
11	Kondaiya	65	M			0	0.01
12	Michel	65	M			0	0
13	Velrajan	29	M			0.02	1
14	Lakshmanan	40	M			4	3.2
15	Gnanamurthi	24	M			0	0

RAGAS DENTAL COLLEGE AND HOSPITAL

Department of Oral Medicine, Diagnosis and Radiology

CASE SHEET PROFOMA

Estimation of cotinine level in the serum and saliva in active smokers and non-smokers

A] General Information

DATE: S.NO. OP. NO.

1. Name:

2. Age:

3. Sex: a. Male b. Female

4. Occupation:

a. Unemployed

b. Professional

c .Administration

d. Trade/Business

e. Student

5. Address:

6. Income:

a. <Rs.1000/month b. > Rs.1000-5000/month c.>Rs.5000 /month

B] Past Dental History:

C] Habit

Smoking: a) Type

b) Duration

c) Frequency

D] Intra oral Examination

Decay:

Missing:

Filled:

Tobacco stains:

E] Investigations

High profile liquid chromatography of

Serum sample

Saliva sample

F] Results

Serum cotinine level

Saliva cotinine level

CONSENT LETTER

I , the under signed hereby give my consent to estimate and compare cotinine level in serum and saliva in smokers and non- smokers by high profile liquid chromatography by Dr. S. Parthiban, under the guidance of Dr.S. Kailasam MDS, Professor and Head of Department of Oral medicine Diagnosis and Radiology, Ragas dental college and Hospital. Chennai. I have been informed and explained about the evaluation procedure, risks involved and likelihood of successes. I also understand and accept this as part of study protocol, thereby voluntarily, unconditionally freely give my consent without any fear or pressure in mentally sound, conscious state to participate in the study.

Witness/Representative Patient Signature

(if any) Date:

ஒப்புதல் படிவம்

---------- என்கின்ற நான், சென்னை ராகாஸ் பல் மருத்துவக் கல்லூரி மற்றும் மருத்துவமனையின் வாய் மருத்துவம் மற்றும் ஊடுகதிர் துறையில் பேராசிரியர் **மரு. S கைலாஸம் B.Sc, M.D.S.,** அவர்களின் மேற்பார்வையில், முதுநிலை (M.D.S) பட்டப்படிப்பு பயிலும் மரு.ச.பார்த்திபன் அவர்கள் மேற்கொள்ளும் ‘‘புகைப்பிடிக்கும் பழக்கம் உள்ளோர் மற்றும் புகைப்பிடிக்கும் பழக்கம் அல்லாதவர்கள் ஆகியோரின் உமிழ்நீர் மற்றும் இரத்த சீரத்தில் கோட்டின் அளவை கண்டறிதல்’’ என்கின்ற ஆராய்ச்சிக்கான பரிசோதனைகளுக்கு என்னை உட்படுத்துவதற்கு எனது மனமுவந்த பரிபூரண சம்மதத்தினை அளிக்கிறேன்.

சாட்சியாளர்கள் : இப்படிக்கு